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The role of the rho-associated coiled-coil containing kinase (ROCK) in cytokine-induced chemokine responses in intestinal epithelial cells

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THE ROLE OF THE RHO-ASSOCIATED COILED-COIL CONTAINING KINASE (ROCK) IN CYTOKINE-INDUCED CHEMOKINE RESPONSES IN INTESTINAL EPITHELIAL CELLS

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Abstract

Inflammatory Bowel Disease (IBD) affects over 1 million Americans and can cause severe tissue damage and even death. TNF-α is a pro-inflammatory cytokine that is found at elevated levels in IBD and plays a central role in inflammation in general. Previous results in our laboratory showed that another pro-inflammatory cytokine, IL-1β, induced chemokine secretion by intestinal epithelial cells (IEC) and that this induction was mediated by the Rho-associated coiled-coil containing kinase (ROCK). Because ROCK may be an important mediator of inflammation in IEC and because chemokines play an important part in the progression of inflammation, we extended our investigations to examine the role of ROCK in TNF-α-stimulated chemokine responses by IEC. Inhibiting ROCK with the Y-27632 compound resulted in a significant, but incomplete, suppression of TNF-α-induced CXCL8 mRNA expression and secretion in Caco-2 cells, indicating that ROCK is required for the optimal CXCL8 production by IEC. ROCK inhibition also blocked TNF-α-stimulated JNK phosphorylation in Caco-2 cells but did not affect IκBα phosphorylation and degradation, suggesting a possible explanation for the partial suppression of CXCL8 as CXCL8 is under the control of both NF-κB and the JNK-activated AP-1 transcription factor. Unlike CXCL8, the production of two other chemokines, CCL2 and CCL20, was enhanced by ROCK inhibition in Caco-2 cells treated with either TNF-α or IL-1β, indicating that ROCK activity had a suppressive effect on CCL2 and CCL20 responses. Further experiments showed that the ROCK-dependent suppression was likely mediated by the ERK pathway since the MEK/ERK

inhibitor, PD98059, had the same enhancement effect as the ROCK inhibitor on TNF-α-induced CCL2 secretion. However, the PI3K/Akt pathway was found not to contribute to the effect. Additionally, inhibiting ROCK significantly reduced ERK phosphorylation in TNF-α-treated Caco-2 cells, showing that ROCK was possibly acting upstream of ERK. These results suggest that ROCK may play an integral and complex role in cytokine-induced chemokine production in IEC. Furthermore, since ROCK appears to control the expression of several pro-inflammatory mediators, ROCK may be a promising therapeutic target for IBD that merits further research.

In loving memory of my grandmother, Nellie VanNostrand

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Abbreviations

AEBSF .....4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride,
a serine protease inhibitor
Akt.....v-Akt murine thymoma viral oncogene homolog,
synonym for protein kinase B
AMP.....Adenosine monophosphate
ANOVA .....Analysis of variance
AP-1 .....Activator protein-1
ARE.....AU-rich element
ASK1.....Apoptosis signal-regulating kinase 1
ATCC.....American Type Culture Collection
ATF.....Activating transcription factor

Bad .....BCL-2-associated agonist of cell death
BCL-2 .....B-cell CLL/lymphoma 2 or apoptosis regulator BCL-2
Bim.....BCL-2-interacting mediator of cell death or BCL-2-like protein 11
BSA.....Bovine serum albumin

CCL.....C-C motif chemokine ligand
CCL2.....A monocyte / macrophage chemoattractant
also known as monocyte chemotactic protein-1
CCL20.....A lymphocyte and DC chemoattractant
also known as macrophage inflammatory protein-3α
CD .....Crohn's disease
$CD4^+$ T cell .....Helper T lymphocyte, positive for the expression
of cluster of differentiation 4 protein
$CD8^+$ T cell .....Cytotoxic T lymphocyte, positive for the expression
of cluster of differentiation 8 protein
Cdc42 .....Cell division control protein 42
CXCL.....C-X-C motif chemokine ligand
CXCL8.....A neutrophil chemoattractant also known as IL-8

DC.....Dendritic cell
DMEM .....Dulbecco's modified Eagle's medium

EDTA.....Ethylenediaminetetraacetate
EGF.....Epidermal growth factor
ELISA .....Enzyme-linked immunosorbent assay
Elk-1.....ETS-like gene 1 or ETS domain-containing protein
ERK.....Extracellular signal-regulated kinase

FBS .....Fetal bovine serum
Fc.....The constant, crystallizable fragment of an antibody produced by proteolytic cleavage
FN .....Fibronectin
Fos.....Homolog of Finkel-Biskis-Jinkins murine osteosarcoma virus oncogene
FOXO4.....Forkhead box protein O4, a transcription factor

GAPDH.....Glyceraldehyde 3-phosphate dehydogenase
GDP.....Guanosine diphosphate
GTP.....Guanosine triphosphate

HRP.....Horseradish peroxidase
HUVECs .....Human umbilical vein endothelial cells

IBD.....Inflammatory bowel disease
IC.....Inhibitory concentration
IEC .....Intestinal epithelial cell(s)
IFN-γ.....Interferon-γ
Ig .....Immunoglobulin (given with a type such as IgA or IgG)
IGEPAL CA-630 .....Octylphenoxypolyethoxyethanol, a nonionic detergent
IκB.....Inhibitor of κ light chain gene enhancer in B cells (e.g. IκBα)
IKK .....IκB kinase
IL.....Interleukin (such as IL-1β)
IL-1Ra.....IL-1 receptor antagonist
IL-1RI, IL-1RII.....Interleukin-1 receptor I or II
ITS.....Insulin, transferrin, and selenium

JIP .....JNK-interacting protein
JNK .....c-Jun N-terminal kinase
c-Jun.....Cellular homolog of v-Jun avian sarcoma virus 17 oncogene

LPA.....Lysophosphatidic acid
LPS.....Lipopolysaccharide

MAPK.....Mitogen-activated protein kinase
MAP2K.....MAPK kinase
MAP3K.....MAP2K kinase
MEK.....MAPK/ERK kinase
MEKK1.....MAPK/ERK kinase kinase 1
MLC.....Myosin light chain

NEMO.....NF-κB essential modulator
NFAT.....Nuclear factor of activated T cells
NF-κB.....Nuclear factor κB
NIK.....NF-κB-inducing kinase

p53.....p53 tumor suppressor or cellular tumor antigen p53
$p90^{RSK}$.....Ribosomal protein S6 kinase
PBS.....Phosphate-buffered saline
PCR.....Polymerase chain reaction
PI3K.....Phosphatidylinositol 3-kinase
PKC.....Protein kinase C
PLSD.....Fisher's protected least significant difference post-hoc test

Rac.....Ras-related C3 botulinum toxin substrate
Raf.....v-raf murine leukemia viral oncogene homolog or
RAF proto-oncogene serine/threonine-protein kinase
Ras.....Rat sarcoma viral oncogene homolog or Ras GTPase
rh.....Recombinant human (as in rhTNF-α or rhIL-1β)
Rho.....Ras homolog gene family member
RhoBTB.....Rho-related BTB domain-containing protein
Rnd.....Rho-related GTP-binding protein
ROCK.....Rho-associated coiled-coil containing protein kinase
RT.....Reverse transcription

SDS-PAGE.....Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM.....Standard error of the mean
siRNA.....Small interfering RNA

TAK1 .....TGF-β-activated kinase 1
TBST.....Tris-buffered saline with 0.1% Tween 20
TGF-β.....Transforming growth factor-β
T_H1, T_H2.....T helper lymphocytes type 1 or 2
T_H17 .....T helper 17 lymphocytes, primary producers of IL-17 and IL-22
TNBS .....Trinitrobenzene sulfonic acid
TNF-α.....Tumor necrosis factor-α
TNFR1, TNFR2 .....Tumor necrosis factor receptor 1 or 2
TRADD.....TNFR1-associated death domain protein
TRAF .....TNFR-associated factor
T_{reg}.....Regulatory T lymphocyte
Tris .....Tris(hydroxymethyl)aminomethane

UC.....Ulcerative colitis

Chapter 1

Introduction: Inflammatory Bowel Disease and the Role of Intestinal Epithelial Cells in the Mucosal Immune System

Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a group of chronic, lifelong gastrointestinal disorders characterized by uncontrolled, relapsing inflammation of the digestive tract [1]. IBD affects approximately 1.4 million people in the Unites States alone and results in more than $1.8 billion in treatment costs annually [2, 3]. While there are several types of IBD, a large majority of cases are diagnosed as either Crohn's disease (CD) or ulcerative colitis (UC) [4, 5]. In CD, inflammation is discontinuous and may be found throughout the gastrointestinal tract, while inflammation in UC is contiguous and confined to the large intestine [1, 5-8]. Inflammation in UC is limited to mucosal and submucosal layers, whereas in CD, it can also affect deeper tissue layers, which accounts for the higher incidence in CD patients of perforations in the gastrointestinal wall and of fistulas, abnormal passages between two gastrointestinal cavities or that open to the skin [9]. UC and CD share several common immediate symptoms, which include fever, abdominal pain, vomiting, and bloody diarrhea. Even when well-managed, IBD can also result in tissue damage leading to ulcerations, perforations, fistulas, or fibrotic adhesions between adjacent sections of intestine [5-7, 10]. In rare cases, toxic megacolon can occur, a life-

threatening condition in which prolonged inflammation causes paralysis, dilatation, and necrosis of the large bowel [11]. Additionally, patients who suffer with IBD for several years have increased risk for intestinal cancers [12].

Despite being the focus of considerable research in recent years, the etiology of IBD is still poorly understood. However, the current consensus is that IBD results from a poorly regulated and inappropriate immune response to commensal intestinal microorganisms [5-8, 13-15]. How this inappropriate response is activated and prolonged is unclear, although the current evidence indicates that the root cause is complex and involves combinations of multiple determinants [5-8, 13-17]. IBD is much more prevalent in industrialized nations [13, 16] and accordingly, was not recognized and described until the early 20th century [18, 19]. Lifestyle / environmental factors that have also been linked to IBD include cigarette smoking, diet, oral contraception, and removal of the appendix [16]. In addition, incidence of IBD has been found to be associated with reduced diversity and other changes in the intestinal flora [6, 15] as well as with variant alleles in more than 70 genetic loci [17, 20].

Along with its fundamental causes, a cure for IBD also remains elusive, and after initial presentation, the disease persists for life, cycling between relapse and remission or, in a small percentage of cases, producing continuous symptoms [9, 21]. Current therapies aim to quell symptoms, to induce and maintain remission, preferably without the use of corticosteroids, and to delay or avoid the need for surgical intervention [22]. Recommended treatment varies depending on the severity of disease, the location and extent of inflammation, the frequency of remission, and other factors [21, 23]. There is a variety of anti-inflammatory and immunosuppressive chemotherapeutic agents used to

treat IBD [9, 21, 23]. However, their efficacy rates for inducing remission range from only 30% to 80% [24, 25]. More recently, biologic therapies have been introduced. Generally speaking, biologics are products derived from or synthesized by living organisms and often are composed of recombinant proteins. For IBD, there are several biologics, many of which target pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α) [25, 26]. These include the anti-TNF-α monoclonal antibodies, infliximab, adalimumab, golimumab, and certolizumab pegol, which have similar efficacies as non-biologics, inducing remission in only 40 – 60% of cases. While long-term use of corticosteroids is considered to carry the highest risk for severe, even life-threatening, side-effects [27], all of these treatments can be accompanied by serious adverse sequelae, including increased risks for opportunistic infections and for certain cancers [21, 23-25]. Furthermore, of those patients responding to the monoclonal antibody biologics, 25 – 70% experience a loss of response to treatment due to the production of anti-drug antibodies [21, 26]. On top of that, despite the goal of preventing the need for surgical treatment, 30 – 40% of ulcerative colitis patients and 70 – 80% of Crohn's disease patients still require at least one surgery during the course of their disease [28].

The current treatments for IBD have many shortcomings, and even those patients who respond well are burdened with relapsing symptoms and lifelong courses of medication. The solutions to these problems lie in finding answers to the many questions that still remain about the causes and progression of IBD. Thus, research that explores the mechanisms of IBD pathogenesis, including the role that intestinal epithelial cells

play in inflammation, must continue if improved treatments and ultimately a cure are ever to be possible.

The Intestinal Mucosa

The innermost layer of the intestine, known as the mucosa or mucous membrane, surrounds the intestinal lumen and interacts directly with its contents. At 200 to 300 m^2, a surface area greater than a tennis court, the human gastrointestinal mucosa is, by far, the most extensive bodily surface to contact the external environment [29, 30]. The mucosa is responsible for nutrient absorption and the secretion of digestive hormones, enzymes, and other substances. In addition to digestive functions, the intestinal mucosa plays a role in immunity by providing a physical, chemical, and immunological barrier to the entry of microorganisms and other pathogenic agents [31].

The overall architecture of the intestinal mucosa differs between the small intestine and the colon [as reviewed in 32]. In the small intestine, finger-like structures called villi project into the lumen, greatly increasing the surface area (Figure 1.1A). Interspersed between the villi are tubular glands called intestinal crypts or crypts of Lieberkühn. In the colon, villi are absent, while numerous closely spaced crypts extend into the colonic surface (Figure 1.1B).

In both the small and large intestines, the mucosa is comprised of three layers: the muscularis mucosae, the lamina propria, and the intestinal epithelium (Figure 1.1). The muscularis mucosae separates the lamina propria from the submucosa and is composed of smooth muscle fibers [32]. The lamina propria is mainly comprised of loose connective tissue interspersed with blood and lymphatic capillaries needed for the transport of

nutrients and hormones [32]. Resident within the lamina propria is a diffuse and diverse population of immune cells including lymphocytes, tissue-specific macrophages, dendritic cells, and granulocytes [33]. Finally, the intestinal epithelium consists of a continuous sheet of columnar epithelial cells that covers the entire intestinal surface, including lining the intestinal crypts. The epithelium serves as a selective barrier, allowing for the absorption of nutrients and physiologically important molecules, while excluding pathological quantities of antigens and organisms [34].

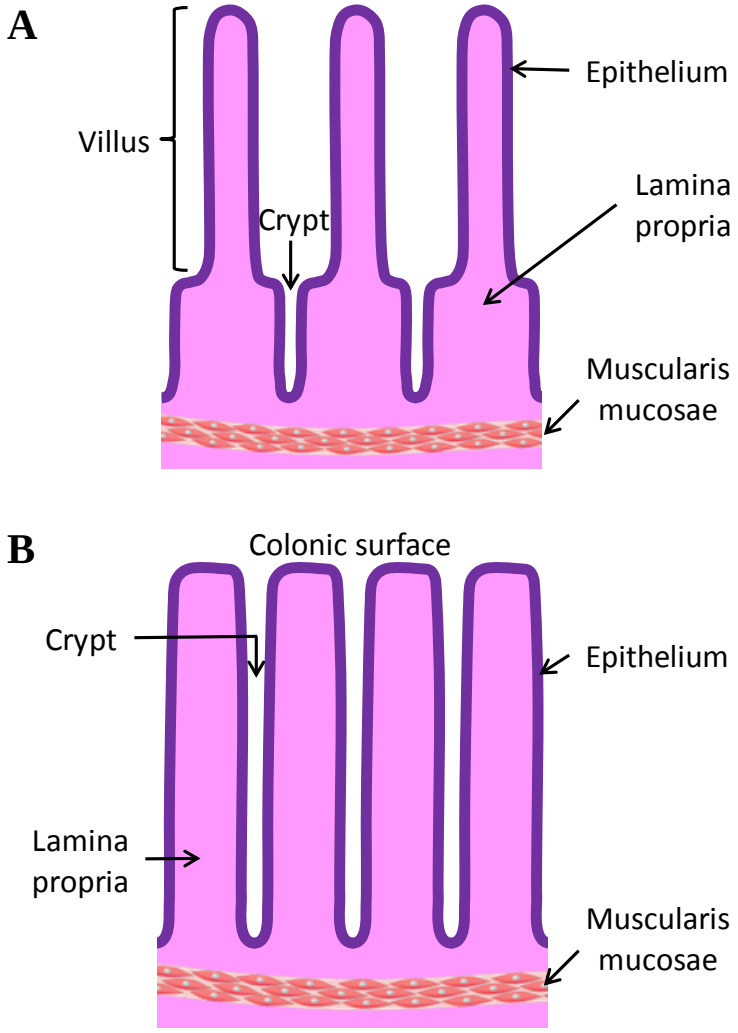

Figure 1.1: An overview of the structure of the mucosa of (A) the small intestine and (B) the colon.

The intestinal epithelium is composed of several cell types that play different roles in intestinal function. These cell types are classified as absorptive epithelial cells (or enterocytes), enteroendocrine cells, goblet cells, or Paneth cells, all of which are derived from intestinal epithelial stem cells found at or near the base of intestinal crypts [35]. Except for Paneth cells, which are located only at the base of the crypt, all other cells derived from intestinal stem cells continually move upward from the crypt and are shed while undergoing apoptosis after reaching the tip of the villus in the small intestine or after reaching the colonic surface. Through this process of rapid turnover, the integrity of the epithelium is maintained despite the harsh chemical environment and frequent abrasion during the digestion and passage of intestinal contents [36, 37]. Epithelial integrity is also maintained by intercellular junctions that tightly bind the cells of the epithelium together, making it highly impermeable to many substances [34]. Because of this, specialized, absorptive intestinal epithelial cells (henceforth referred to as IEC) are required for nutrient uptake, which is performed via transcellular transport in the case of small soluble molecules and vesicular transcytosis in the case of larger factors [32, 34]. Additionally, IEC of the small intestine have direct involvement in digestion, expressing membrane-bound hydrolytic enzymes [32]. Also present in the epithelium are enteroendocrine cells that help regulate digestion and gastrointestinal motility by producing hormones and other signals such as secretin, cholecystokinin, and serotonin [38]. The third cell type is the goblet cell. The main function of the goblet cell is the production of mucins, glycoproteins that are converted to mucus upon secretion [39]. Mucus forms a layer covering the epithelium, lubricating the movement of luminal contents through the digestive tract, as well as providing a physical buffer that allows the

diffusion of nutrients while limiting direct contact between the epithelium and luminal organisms and antigens [32]. Lastly, Paneth cells provide protection and help regulate the growth of the epithelium. In defense against bacterial invasion, Paneth cells secrete many protective substances into the intestinal lumen, including antimicrobials, like lysozyme [40], α-defensins, which are proteins that form pores in bacterial plasma membranes [41], and TNF-α, an important inducer of inflammation [42, 43]. Normally, Paneth cells are found only in the crypts of the small intestine in close proximity to intestinal epithelial stem cells, but they can also be detected in colonic crypts during disease [41].

The Immunological Function of Intestinal Epithelial Cells

In addition to absorptive and physical barrier functions, IEC also play an important role in immunity. As the front-line in the intestinal mucosa, IEC are the first cells to encounter invading pathogens, and thus, IEC must be able to initiate immune responses to allow for early detection and control of infection. IEC sense pathogen invasion and infection via pattern recognition receptors [44]. These receptors detect pathogen-associated molecular patterns, which are molecular components, such as peptidoglycan, that are characteristic of large groups of pathogens. Additionally, IEC express receptors for several cytokines, which are low-molecular-weight peptide signaling molecules that mediate communication between and regulate the activity of cells participating in immune responses [45]. Among the receptors expressed are those for interleukin (IL)-1, IL-6, IL-17, TNF-α, and Transforming Growth Factor-β (TGF-β)

[46]. The expression of these receptors allows for the regulation of IEC immunological responses and for IEC to take part in immune system signaling networks.

Upon encountering foreign antigens or pro-inflammatory cytokines, IEC respond in several ways that can advance the inflammatory response or otherwise aid in innate and adaptive immunity. IEC can produce the antibiotic peptides, β-defensins, and have been found to secrete many cytokines such as IL-1β, TNF-α, and IL-6. In response to pro-inflammatory signals, IEC also produce several chemokines, a sub-class of cytokines that attract and activate leukocytes [47-51]. Those leukocytes can then direct further immunological responses. Additionally, IEC can play a role as antigen-presenting cells, expressing major histocompatibility complex class II proteins, co-stimulatory molecules, and adhesion molecules required for the activation of T cells [as reviewed in 52].

The Inflammatory Response

Upon injury and/or in the beginning stages of infection, the innate immune system mounts an acute inflammatory response [as reviewed in 45]. This response is a cascade of protective measures, the goal of which is to control and clear infection while initiating the healing process. Early in this response, localized vasodilation and increased vascular permeability allow for the influx of fluid from the capillaries into the affected area and results in the characteristic edema, heat, and redness of inflammation. After vascular changes have begun, neutrophils begin to infiltrate the site of inflammation, followed later by monocytes and lymphocytes. In order for infiltration to occur, circulating leukocytes must be able to exit the bloodstream and home in on the affected area. Chemotactic chemokines, produced in response to IL-1, TNF-α, and other inflammatory

signals, recruit leukocytes from the bloodstream, which then follow the chemokine gradients to the site of inflammation. After infiltration, the various types of leukocytes work in concert with cells already resident at the affected site to mount a wide array of immunological responses.

The first of the leukocytes to arrive at a new site of inflammation are the neutrophils, professional pathogen killers that remove invading organisms by phagocytosis and produce many antimicrobial substances [as reviewed in 53]. Upon activation at a site of infection, neutrophils undergo the process of respiratory burst in which oxygen uptake greatly increases leading to the production of multiple toxic reactive oxygen species, reactive nitrogen species, and hypochlorite. Activated neutrophils also undergo degranulation in which the contents of storage vesicles are released into the extracellular milieu or into phagolysosomes, large vesicles containing phagocytosed microbes. Neutrophil granules contain many antimicrobial substances, which include proteins that can permeabilize microbial plasma membranes such as bactericidal/permeability increasing protein, β-defensins, and cathelicidin. Neutrophils also secrete proteases, which, in addition to attacking invaders, digest extracellular matrix and surface proteins on cells in the surrounding tissues.

While neutrophils have a singular purpose, macrophages take on a wider range of roles in acute inflammation. Macrophages can be stimulated by various combinations of interferon-γ (IFN-γ), TNF-α and microbial products. Like neutrophils, macrophages produce a wide array of pro-inflammatory cytokines and are believed to be the main source of the major inflammatory mediators, IL-1, TNF-α, and IL-6, during acute inflammation [54, 55]. In addition, macrophages interact with and activate antigen-

specific T cells, which then produce additional cytokines that further the immune response [54, 56].

In order to defend against the full array of pathogens the human body can encounter, the adaptive immune system is also activated during inflammation, widening the variety of possible responses. One of the major types of cells in the adaptive immune system is $CD4^+$ T lymphocytes (helper T cells), of which there are several subtypes. Once activated, helper T cells play central roles in the regulation and progression of both innate and adaptive immune functions. In cell-mediated immunity, type 1 helper T cells (T_H1) activate and orchestrate the activities of several other cell types, including neutrophils and macrophages (hence the term cell-mediated) [45, 57]. In contrast, immune functions governed by type 2 helper T cells (T_H2) are termed humoral and are characterized by the increased production of antibodies by B cells and the activation of eosinophils [57, 58]. Among the other subsets of $CD4^+$ T cells are regulatory T cells (T_{reg}), which act to limit inflammation by secreting large amounts of anti-inflammatory IL-10, and also inflammatory T helper 17 (T_H17) cells [59]. T_H17 cells characteristically produce the pro-inflammatory cytokine, IL-17, and play an important role in the progression of inflammation and other immune responses during infection by extracellular bacteria and fungi [57, 59, 60]. Interestingly, it is because of this role in inflammation that T_H17 cells have been implicated in several autoimmune disorders, including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and IBD [61].

The Histological and Cellular Pathology of Inflammatory Bowel Disease

Numerous functional abnormalities and morphological changes in IBD-affected tissues and cells have been identified [62-67]. In the mucosae of IBD sufferers, IEC exhibit poorly formed microvilli, and the normally smooth surface of the epithelium has an irregular appearance [67, 68]. In inflamed tissues, damage to the epithelium due to increased IEC apoptosis is repaired through restitution, a process in which IEC rapidly spread and move into damaged or denuded areas to close any gaps [67, 69-71]. Restitution occurs independently of cell proliferation and on a much quicker time scale. Consequently, as the IEC compensate to cover more area and maintain epithelial integrity, the thickness of the epithelium is reduced, and the IEC lose their columnar shape and become somewhat cuboidal [66-68, 72]. Also accompanying these changes are a reduction or loss of the protective mucus layer, an increase in epithelial permeability, and often, the abnormal presence of Paneth cells in the colonic epithelium [41, 66, 67]. If inflammation persists unchecked, continued damage to the epithelium outstrips the ability of restitution and IEC proliferation to maintain the epithelial barrier and erosion of the epithelium occurs, leading to ulcerations involving subepithelial and, potentially, submucosal tissue layers [66, 67].

As previously described, the normal, non-inflamed intestinal mucosa contains a diverse population of leukocytes, including dendritic cells, granulocytes, tissue-specific macrophages, and various subsets of mucosal-homing lymphocytes. Under normal conditions, these leukocytes are responsible for immune surveillance, maintaining tolerance to intestinal microbiota, and activating defenses against pathogens when needed. However, in IBD, there is a marked increase in the number of immune cells in

the mucosa due to an influx of activated, pro-inflammatory leukocytes [6, 73]. This influx includes neutrophils, eosinophils, mast cells, macrophages, plasma B cells, as well as CD- or UC-specific profiles of effector T lymphocytes (T_H1, T_H2, T_H17, T_{reg}, $CD8^+$ cytotoxic T cells, and natural killer T cells) [6, 7, 74]. These cells are targeted mainly to the lamina propria [6]. However, during active inflammation, neutrophils are also found within the epithelium and often transmigrate into the intestinal lumen and particularly into the crypts where they can cause cryptitis and crypt abscesses, both of which are indicators of advanced inflammation [66, 67, 75]. With the increased numbers of activated leukocytes in the mucosa also comes the increased production of several pro-inflammatory cytokines, including chemotactic chemokines [62], IL-1β, and TNF-α [76-78].

IL-1β and TNF-α: Major Mediators of Inflammation

The cytokine, Interleukin-1, plays an important and central role in the initiation and progression of immune responses. Interleukin-1 consists of two isoforms coded by two homologous genes: *IL-1α* and *IL-1β*. IL-1α is produced by many cell types as either a membrane-bound or cytoplasmic protein and is rarely released into the extracellular fluid, except when cells undergo necrosis or rupture [79, 80]. Thus, IL-1α is mainly involved in the immune response to cell death from non-pathogen-related causes such as trauma or ischemia [79, 80]. In contrast, IL-1β is a secreted protein that plays a central role in the response to pathogens and is a major mediator of inflammation in general [79-82]. While macrophages are the main source of IL-1β, secretion of IL-1β has also been observed in IEC, dendritic cells, microglia, fibroblasts, smooth muscle cells, and several

other cell types [50, 79-81]. Not surprisingly, considering its important role in inflammation, IL-1β has been found to be involved in numerous autoimmune and autoinflammatory diseases [79-83].

As it is pleiotropic, IL-1β has been found to elicit a wide array of local and systemic pro-inflammatory and pro-healing effects [79-83]. In target cells, IL-1β stimulation results in the expression of a multitude of genes [81-83]. Among these genes are those that code for matrix metalloproteinases responsible for the degradation of the extracellular matrix and the resorption of bone and cartilage that can occur during prolonged inflammation [79, 81-83]. IL-1β also induces the expression of surface adhesion molecules, such as vascular cell adhesion molecule-1 by endothelial cells, which are required for the extravasation of leukocytes from the bloodstream [79-83]. Perhaps more importantly, however, IL-1β initiates a cascade of inflammatory signals by inducing the expression of several other cytokines, including chemokines [79-83], IL-6 [79, 84], IL-23 and IL-17 [79-81], and even IL-1β itself [79, 80]. Some of these cytokines can, in turn, stimulate the production of further downstream cytokines, while some are involved in the differentiation, proliferation, and activation of other leukocytes [79-81]. Furthermore, IL-1β is also involved in inducing fever and in angiogenesis and can indirectly cause the release of corticosteroids involved in stress response [79, 80, 83].

While it is clear that IL-1β is an important pro-inflammatory mediator, it is TNF-α that truly plays a central role and has even been termed the “master regulator” of inflammation [85]. TNF-α is produced as a membrane-bound homotrimeric protein that is released into the intercellular milieu by proteolytic cleavage [86]. Macrophages are thought to be the main source of TNF-α in the human body [86, 87]. However, under

pro-inflammatory conditions, several other cells types are known to produce TNF-α, including IEC [50]. TNF-α is a pleiotropic cytokine with many different effects in a wide spectrum of physiological contexts. Perhaps most importantly, TNF-α plays a critical role in the defense against pathogens [86, 88]. Because of this role in the response to pathogens, TNF-α has also been implicated in septic shock with some studies showing TNF-α to be the primary driver of host damage [86, 88]. TNF-α has been also been implicated in several other physiological processes and conditions, including cachexia, liver destruction during acute hepatotoxicity, rheumatoid arthritis, IBD, and, under certain circumstances, it can cause tumor necrosis [86, 88, 89].

This profusion of physiological activities arises from both the direct and indirect effects that TNF-α exerts on a wide array of cell types. These effects are mediated by two TNF-α-binding receptors: TNFR1 and TNFR2. Expression of TNFR2 is highly regulated and is only found in a limited number of cell types, including endothelial cells, thymocytes, T lymphocytes, and astrocytes, whereas TNFR1 is expressed ubiquitously and in a constitutive manner [86, 90]. It is this pervasive expression of TNFR1 that accounts, at least in part, for the pleiotropy that TNF-α possesses, and the binding of TNF-α to its receptors can evoke a multitude of cellular responses. For instance, under certain cellular contexts, TNF-α can induce cell death through either apoptosis or necroptosis [87, 90], while more commonly, TNF-α induces the expression of a range of genes involved in such processes as cell proliferation, cell survival, and the production of pro-inflammatory cytokines [86, 87].

Not surprisingly, there is a complex regulatory relationship between TNF-α and IL-1β, each affecting the action of the other in various ways. For example, neutralization

of TNF-α with antibodies was found to significantly reduce the production of IL-1β in cultures of synoviocytes isolated from rheumatoid arthritis patients, indicating that TNF-α induces IL-1β secretion in these cells [91]. TNF-α has also been found to affect IL-1 signaling by downregulating IL-1 receptor I (IL-1RI) expression in the rat IEC-6 cell line [92], while it was found to upregulate both IL-1β production and IL-1RI expression in bone marrow stromal cells [93]. Furthermore, TNF-α and IL-1β are known to act together synergistically [94], for instance, in the production of cytokines in IEC [95-97].

Chemokines

Chemokines are a superfamily of cytokines that consist of small (~8 – 14 kDa), soluble, heparin-binding proteins that have several functions in host defense and maintenance of the immune system [98]. The chemokine superfamily has been classified into four families based on the configuration of two highly conserved N-terminal cysteine residues [98]. These are the CC, CXC, CX_3C, and C families. The CX_3C and C families are minor groupings with one and two members, respectively, whereas the CC and CXC families contain the bulk of the more than fifty chemokine members [98, 99]. While there is no hard rule regarding what leukocytes are affected by any given family of chemokines, neutrophils are mainly acted upon by CXC chemokines, while macrophages and monocytes are predominantly acted upon by CC chemokines [100, 101]. Furthermore, chemokines are promiscuous, some interacting with more than one receptor and most affecting multiple cell types [100].

Many chemokines are directly involved in inflammation, while some have functions in immune system homeostasis and other processes [as reviewed in 98, 99, 100, 101]. Generally speaking, chemokines guide leukocyte traffic by acting as chemoattractants. For pro-inflammatory chemokines, this is their primary purpose, drawing leukocytes to sites of infection or inflammation. Due to the transient nature of host defense, pro-inflammatory chemokines are expressed as needed and are induced by host signals, like TNF-α, or by the detection of foreign antigens. In addition to providing leukocytes direction to sites of inflammation, pro-inflammatory chemokines also play a key role in the extravasation of leukocytes from the bloodstream by inducing the activation of integrins on the surface of leukocytes, which allows the leukocytes to attach to adhesion molecules expressed by vascular endothelial cells and then exit the blood vessel via diapedesis [62, 101]. Some chemokines also further the inflammatory response by stimulating leukocytes to release of hydrolytic enzymes or to undergo oxidative burst, the generation of reactive oxygen species and reactive nitrogen species [45, 62].

The first chemokine to be discovered was CXCL8, also known as interleukin-8 [102]. It was originally found to be chemotactic for neutrophils. However, it has also been reported to induce homing in monocytes, basophils and $CD4^+$ and $CD8^+$ T cells as well [103-105]. Secretion of CXCL8 is highly regulated, and little to none is generated unless cells are stimulated [106]. When stimulation does occur, production of CXCL8 increases rapidly, and large amounts can be secreted in a relatively short time [106, 107]. Stimuli that induce CXCL8 expression include cytokines, such as TNF-α and IL-1β, pathogen-associated molecular patterns such as lipopolysaccharide (LPS), and cellular

stress [106]. CXCL8 is widely produced and has been observed in IEC, monocytes, neutrophils, endothelial cells, and many others [106, 108]. In addition to inducing homing, CXCL8 also acts as an activator of neutrophils, prompting vascular adhesion and extravasation, degranulation, and respiratory burst [45, 103]. In addition, CXCL8 was the first chemokine found to be angiogenic [109] and has been shown to promote re-epithelialization in cutaneous and intestinal wounds [101, 108, 110, 111].

CCL2, previously known as monocyte chemotactic protein-1, is another important chemokine. Like CXCL8, CCL2 is expressed by a wide variety of cells including IEC [62], other epithelial cells, fibroblasts, smooth muscle cells, endothelial cells, astrocytes, and monocytes [112]. Production of CCL2 is also induced by a similar profile of stimuli to that which induces CXCL8, which includes TNF-α, IL-1, IL-4, IFN-γ, LPS, and cellular stress [112]. Perhaps the most important chemotactic targets of CCL2 are macrophages and monocytes, which are also a major source of this chemokine [112]. In addition, CCL2 also recruits natural killer cells, activated T cells, and immature dendritic cells to sites of inflammation [62] and can participate in angiogenesis by stimulating endothelial cell migration and inducing the expression of vascular endothelial growth factor [112].

Whereas some chemokines are inducible and pro-inflammatory, like CXCL8 and CCL2, and some are constitutively produced and homeostatic, a few chemokines are both [101]. One such example of a dual-capacity chemokine is CCL20, which was previously known as macrophage inflammatory protein-3α. CCL20 is chemotactic for T cells, B cells, and immature dendritic cells [101]. Generally, it is expressed constitutively in and near secondary lymphoid tissues, but not in primary lymphoid tissues or the spleen,

which is indicative of its role in the trafficking of unactivated leukocytes to areas of immune surveillance [113]. In the small intestine, IEC produce CCL20 in order to direct immature dendritic cells and lymphocytes to the subepithelial dome of the Peyer's patches and of lymphoid follicles and to the lamina propria in general [101, 113]. Under inflammatory conditions, CCL20 is produced not just at higher levels by cells that normally express this chemokine but also by many inflammation-related cell types, including endothelial cells, neutrophils, macrophage, dendritic cells, natural killer cells, B cells, and T_H17 cells [113-115]. As this array of cell types would suggest, CCL20 is induced by a wide variety of pro-inflammatory mediators, such as TNF-α, IL-1β, IL-17, and IFN-γ [113].

Cytokines in IBD

One of the primary characteristics of IBD is the aberrant production of pro-inflammatory cytokines [116]. Because of its central role in inflammation, the cytokine TNF-α has received much attention, and several studies have measured TNF-α in IBD patients. Elevated levels of TNF-α have been detected in stool [117, 118] and blood serum [119] from patients with active disease. Plus, increased numbers of TNF-α-producing cells have been found in IBD-affected tissues [120, 121] with TNF-α-positive cells located mainly in the lamina propria and in the submucosa [121]. More commonly, studies have measured TNF-α secretion by enzyme-linked immunosorbent assay (ELISA) and have found significantly elevated levels of TNF-α spontaneously produced by cultured biopsies from IBD patients [77, 122-124] and by lamina propria mononuclear cells isolated from IBD patients [78]. Some of those studies also found elevated levels of

the pro-inflammatory cytokines IL-1 and IL-6 in IBD specimens [77, 78, 122], although McCormack et al. [124] only found elevated levels of TNF-α and IL-6 in CD-affected, but not UC-affected, tissues. Moreover, elevated levels of TNF-α, IL-1β, and IL-6 production have been found even in non-inflamed tissues of IBD patients [77, 78], suggesting that cytokine imbalance may be a prerequisite for active disease. This imbalance also has implications in the progression of IBD, as the number of TNF-α-producing cells has been found to correlate with the severity of inflammation in CD [120]. Additionally, increased levels of TNF-α and IL-1β were measured in the rectal mucosal fluid of IBD patients, and levels of each also correlated separately with sigmoidoscopic scores, clinical scores, and histological scores of disease activity [125].

Biologics are the newest generation of drugs. Not surprisingly, those that are used in the treatment of IBD target the aberrant production of cytokines, particularly TNF-α. The currently approved drugs are mainly recombinant anti-TNF-α monoclonal antibodies and are believed to work by neutralizing soluble TNF-α, by inducing apoptosis in activated inflammatory cells, and by increasing the production of anti-inflammatory molecules [126]. The biologics approved for use in the United States are infliximab, adalimumab, certolizumab pegol, and golimumab [127]. Another anti-TNF-α agent, etanercept, which is a fusion protein consisting of the binding portion of the TNF receptor 2 (TNFR2) and the Fc domain of the IgG1 antibody, failed clinical trials for IBD despite being approved for rheumatoid arthritis and having a similar efficacy as adalimumab for that disease [126]. Aside from targeting TNF-α, two biologics that target integrins, natalizumab and vedolizumab, have been approved [127]. These bind to the α4 integrin subunit and the α4β7 integrin, respectively, and block the vascular adhesion and

extravasation of lymphocytes, preventing the progression of inflammatory infiltrate into intestinal tissues. In addition, several treatments that target cytokines other than TNF-α are currently approved or are in development. These include the IL-12- and IL-23-targeting ustekinumab, which was recently approved for the treatment of CD [128]; tocilizumab that targets IL-6, which is in clinical trials; and four agents that target IL-13, also in trials [127].

The anti-TNF-α therapies listed above all have similar efficacies and induce remission in 40 - 60% patients [23, 25, 26, 126], and similar rates of remission have been reported for the anti-IL-12/IL-23 ustekinumab as well [129]. Unfortunately, this leaves approximately 50% of patients as primary non-responders, although lack of response to one agent does not necessarily predict a lack of response to others [23]. In addition, secondary loss of response also occurs in 70% of those who initially respond to the anti-TNF-α drugs [26]. This is due to the immunogenicity of these recombinant proteins, which leads to the formation of anti-drug antibodies by the patients' immune systems. Furthermore, in the case of infliximab, pre-existing anti-drug antibodies have been found in naïve patients [130], which may explain at least a percentage of primary non-responders and suggests the possibility of antidrug antibodies to other agents as well. Despite these shortcomings, these agents are efficacious for many patients, yet are not without potential side effects, such as anaphylaxis [23], increased risk of opportunistic infections [9, 23], and increased risk for hepatosplenic T cell lymphoma in young patients [131]. Clearly, these treatments are not a panacea, and more research is needed if they are to be improved of if new therapies are to be developed.

Pro-inflammatory cytokines are not alone in their dysregulation in IBD. Abnormal production or dysfunction in the signaling mechanisms of several anti-inflammatory mediators is also found in IBD. For example, IL-1 receptor antagonist (IL-1Ra) is a soluble protein that attenuates IL-1 signaling by competitive binding to IL-1RI [81]. In IBD, increased levels of IL-1Ra have been found in mucosal biopsy specimens [132] and in stool samples [118]. More significantly, however, the ratio of IL-1Ra to IL-1 was shown to be greatly reduced in both mucosal biopsies and the stool from IBD patients, suggesting an increased rate of unimpeded IL-1 signaling in these patients. Furthermore, one polymorphism of the *IL-1Ra* gene has been associated with a higher risk for UC [133]. Other anti-inflammatory mediators that are dysregulated in IBD include the soluble forms of TNFR1 and 2, which are formed by proteolytic cleavage of the extracellular domains of these receptors [134]. Once cleaved, the soluble forms then act as decoy receptors for TNF-α. Accordingly, serum concentrations of both soluble receptors are elevated in both CD and UC [134, 135] and correlate with disease activity [134]. Another anti-inflammatory mediator is the cytokine IL-10, which downregulates antigen presentation and pro-inflammatory cytokine secretion in leukocytes [116]. Measurements of serum levels for IL-10 showed increases of IL-10 in patients with active disease [136], and one study found that expression of IL-10 mRNA was upregulated in intestinal tissues from patients with active CD and UC as well as inactive UC [137]. In addition, loss-of-function mutations in the IL-10 receptor have been associated with early onset of IBD in children [138]. Similarly, IL-10 knockout is sufficient for the development of chronic intestinal inflammation in mouse models of IBD [116]. The anti-inflammatory cytokine TGF-β1 also exhibits defective signaling in

IBD [116, 139]. Tissues and cells isolated from IBD patients are insensitive to TGF-β1 and treatment with TGF-β1 fails to elicit normal inhibition of the production of pro-inflammatory cytokines [139]. Despite these findings, it is still unclear what functional consequences the aberrant regulation of these anti-inflammatory mediators has in the pathogenesis of IBD, although some work has been done on developing IL-10 as a treatment [140], and a therapy to restore TGF-β1 signaling is currently undergoing trials [127].

Chemokines are also found to be dysregulated in IBD. It is believed that, generally speaking, chemokines contribute to the pathophysiology of IBD by promoting the activation and influx of leukocytes into intestinal tissues [62]. The increased numbers of inflammatory cells then produce further chemokines that draw additional leukocytes, resulting in a positive feedback loop. Many chemokines are found at elevated levels in IBD, and among them are the neutrophil-attracting CXCL1, CXCL2, CXCL5, CXCL6, and CXCL8 chemokines [14, 141]. Also included are monocyte/macrophage attractants, such as CCL2, CCL3, CCL4, CCL5, CCL7, and CCL8 [14, 62, 74]. Several of the latter group also induce homing in T cells, B cells, and dendritic cells as well [62]. CXCL8 is of particular importance and has been the focus of much study. In addition to overall increased production of CXCL8 in IBD [74, 141-143], one study found increased expression of CXCL8 mRNA specifically in ulcerated regions of resected bowel specimens [144]. Furthermore, levels of CXCL8 have been found to correlate with the severity of inflammation in IBD [143, 144] and, as would be expected, to correlate highly ($r = 0.95$) with neutrophil numbers in colonic tissues of IBD patients [143]. For CCL2, increased mRNA expression and protein production has been found in macrophages,

smooth muscle cells, and endothelial cells of the lamina propria and submucosa of IBD-affected intestinal tissues [145, 146], as well as in IEC [146]. Another chemokine that is upregulated in IBD is CCL20, which is normally homeostatic, constitutively expressed, and attracts T cells and dendritic cells [62, 101, 141]. Experiments using both fluorescent confocal microscopy [114] and immunohistochemistry [115] have shown that IEC are the main source of CCL20, expressing the protein in normal tissues and at elevated levels in inflamed tissues. Furthermore, CCL20 protein levels in tissue homogenates were found to be elevated in patients with active CD and UC as well as from patients with inactive CD [115].

IEC in inflammation

Not only does the intestinal epithelium form a physical barrier to the entry of bacteria and other luminal organisms into the body, but the cells of the epithelium also act as direct participants in immune responses. *In vitro* experiments with IEC have shown that infection with intracellular pathogens stimulates the production of chemokines by IEC [48, 147]. Furthermore, IEC express several pattern recognition receptors, including toll-like receptors and nucleotide-binding oligomerization domain protein 2, which allow IEC to directly sense the presence of pathogen components [44]. After binding of their respective ligands, these receptors subsequently activate the transcription factors nuclear factor κB (NF-κB) and activator protein-1 (AP-1), among others, and induce the expression of pro-inflammatory genes, although some toll-like receptor activity has been linked to immune tolerance as well [44]. In addition to directly reacting to luminal organisms, IEC also participate in the immune system by responding

to many cytokines. One study showed that both isolated normal rat IEC and the IEC-6 rat cell line expressed IL-1RI mRNA, but not the negative regulator IL-1RII, which is a decoy receptor [92]. Additionally, Panja *et al.* [46] found evidence that IEC isolated from normal human intestinal specimens express receptors for IL-1 as well as for IL-6. That group also found evidence for TNF receptor expression in HT-29, DLD-1, and Caco-2 intestinal epithelial cell lines. Similarly, Mizoguchi *et al.* [148] found that IEC in normal human tissues express TNFR1, but not TNFR2, while IEC in samples from CD and UC did express TNFR2. Furthermore, that study also showed that the expression of both TNFR1 and 2 was upregulated in actively inflamed tissues in a mouse model of colitis.

IEC react to pathogen challenge or to stimulation with cytokines by upregulating many genes involved in inflammation, including additional cytokines and adhesion molecules [147]. For instance, when infected with enteroinvasive bacteria, both the Caco-2 and the HT-29 IEC cell lines have been found to secrete increased amounts of CXCL8 and CCL2 [50, 51]. Infected HT-29 cells have also been shown to produce CXCL1, CCL4, and CCL5 [51] as well as TNF-α and granulocyte/monocyte colony stimulating factor [50]. The HT-29 and T84 cell lines have also been shown to produce increased levels of CCL20 in response to bacterial invasion [114]. This array of cytokine/chemokine responses to bacteria suggests that IEC play an important part in the inflammatory response versus pathogens. IEC also participate in the progression of inflammation by responding to pro-inflammatory cytokines, particularly IL-1β and TNF-α. A third important pro-inflammatory cytokine, IL-6, is produced by the Caco-2 and CCL-241 IEC cell lines when treated with IL-1β or TNF-α [149, 150], and IL-6

secretion is increased synergistically in IEC-6 and Caco-2 cells when treated with IL-1β and TNF-α together [96, 97]. Similar to their response to bacterial infection, IEC also produce a number of chemokines in response to pro-inflammatory cytokines. For example, the Caco-2 and HT-29 cell lines are known to secrete CXCL8 when stimulated with IL-1β or TNF-α [151] or both [51]. Interestingly, and likely not coincidentally, increased levels of CXCL8 have been found to correlate with increased levels of both IL-1β and TNF-α in IBD-affected tissues [143]. In addition to CXCL8, HT-29 and Caco-2 cells have been shown to secrete or express mRNA for CXCL1, CXCL3, CXCL5, CXCL10, CCL2, CCL4, CCL5, and CCL7 when treated with TNF-α [46, 50, 51]. Elevated amounts of CCL20 are also produced by HT-29, Caco-2, and T84 cells in response to either IL-1β or TNF-α [114]. Furthermore, one study examined IEC in intestinal biopsies by immunohistochemistry and found increased expression of CXCL8, CCL2, CCL3, CCL4, and CCL7 [74], while another study found CCL20 to be upregulated by IEC in inflamed human mucosae [114].

TNF-α and IL-1 signaling

TNF-α signaling is highly complicated and involves the activation of a network of intracellular signaling pathways that have not yet been fully elucidated. Similar to other protein ligands, transduction of the TNF-α signal begins with either of two transmembrane receptors: the ubiquitously expressed, death-domain-containing 55 kDa TNFR1 and the 75 kDa TNFR2, which has limited expression (Figure 1.2) [87, 90]. Upon binding of TNF-α to either receptor, assembly of a receptor signaling complex is initiated and proceeds through the sequential recruitment of several adapter proteins, non-

degradative ubiquitin ligases, and kinases [as reviewed in 87]. For TNFR1, the primary event in the assembly of the complex is the direct binding of the TNFR1-associated death domain protein (TRADD) to the death domain of TNFR1. This allows for the recruitment of TNFR-associated factor 2 (TRAF2) and TRAF5. In TNFR2 signaling, TRAF2 and TRAF5 bind directly to the activated receptor. The binding of additional proteins to TRAF2 and TRAF5 follows and leads to the downstream activation of TGF-β-activated kinase 1 (TAK1) as well as the recruitment of the IKK (inhibitor of κ light chain gene enhancer in B cells (IκB) kinase) complex. TAK1 can then initiate NF-κB signaling by phosphorylating and activating IKKα and IKKβ, which, in turn, phosphorylate IκB proteins.

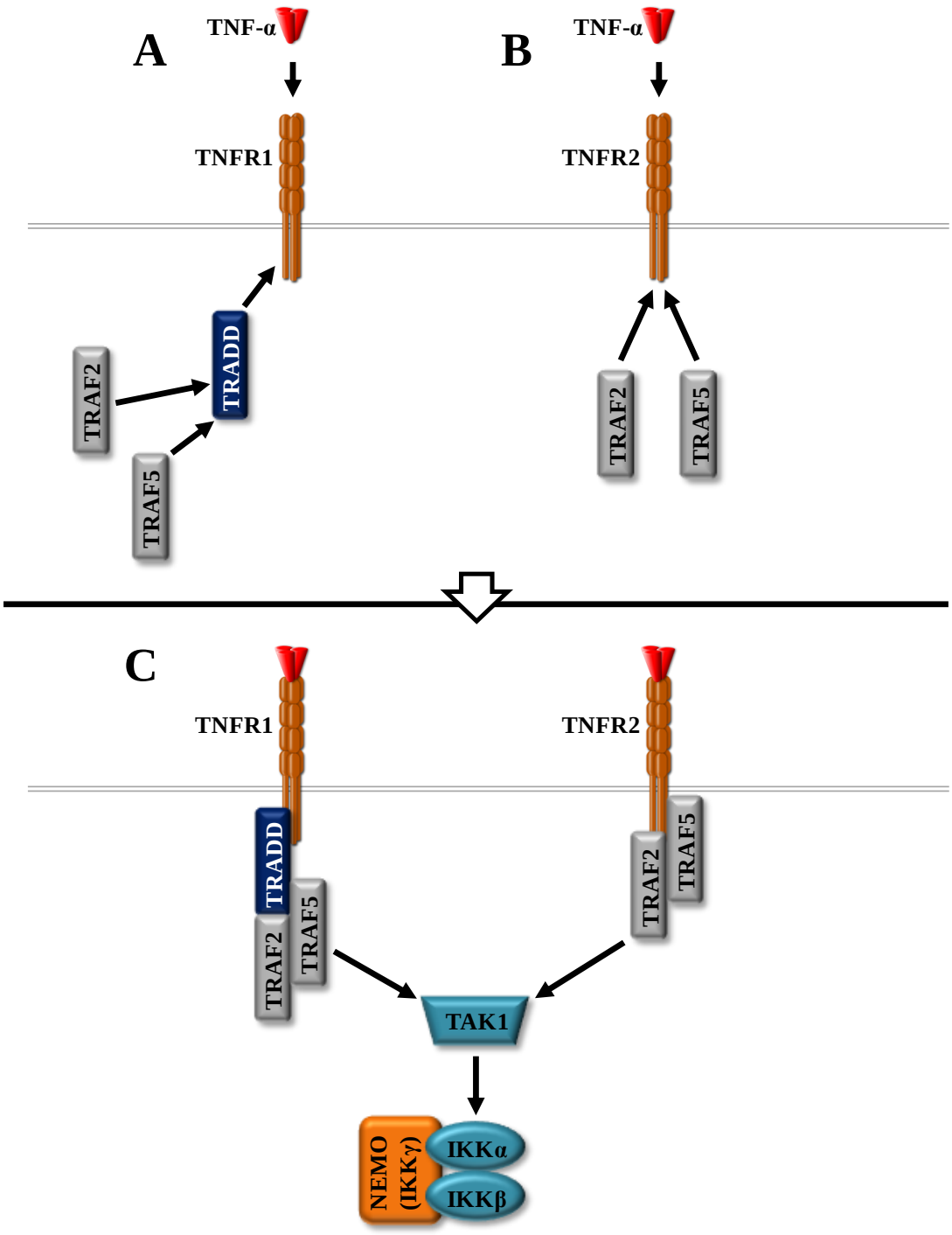

Figure 1.2: The initial stages of TNF-α signal transduction. **A**) When TNF-α binds to the TNFR1 receptor, the resulting conformational change in the receptor initiates the formation of a complex that starts with the recruitment of TRADD, which, in turn, recruits TRAF2 and TRAF5. **B**) Formation of the TNFR2 receptor complex proceeds similarly to TNFR1 signaling except that TRAF2 and TRAF5 bind directly to the activated receptor. **C**) Formation of either receptor complex leads to the activation of the TAK1 kinase which then activates the IKK complex by phosphorylating IKKα and IKKβ.

Aside from the NF-κB pathways, TNF-α is also known to activate several other signaling pathways (Figure 1.3). After its TNF-α-induced activation, TAK1 may also participate in additional downstream events and may act as the mitogen-activated protein kinase kinase kinase (MAP3K) for either the c-Jun N-terminal kinase (JNK) pathway or the p38 mitogen-activated protein kinase (MAPK) pathway, which are both activated by TNF-α [86, 87]. However, the exact role of TAK1 is as yet unclear, and it may be that TAK1 acts further upstream as an activator of other MAP3Ks, such as MAPK/ERK kinase kinase 1 (MEKK1) or apoptosis signal-regulating kinase 1 (ASK1) [86, 90]. The ERK (extracellular signal-regulated kinase) pathway is also activated by TNF-α, albeit in some circumstances, it seems this activation may be indirect via an autocrine loop [86, 152]. Additionally, there have been reports of TNF-α inducing the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [153, 154].

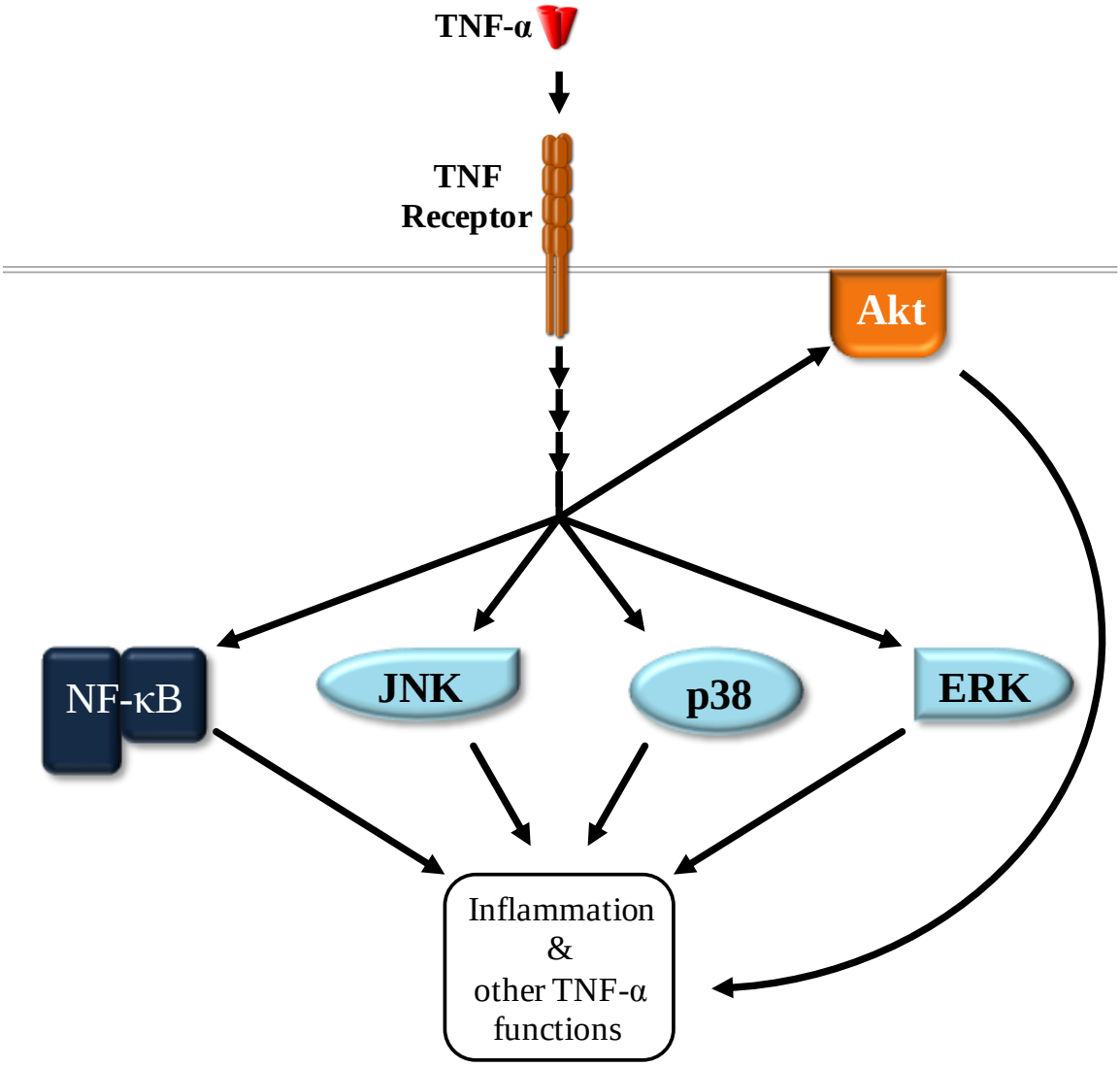

Figure 1.3: Signaling pathways activated by TNF-α. TNF-α signaling is highly complex and results in the simultaneous activation of multiple signaling pathways. These include NF-κB and the three major MAPK pathways: JNK, p38 MAPK, and ERK. Under some conditions, TNF-α also activates the PI3K/Akt pathway.

IL-1β signaling is similarly complex, and many of the same pathways are activated as with TNF-α. In target cells, IL-1β stimulation results in the simultaneous activation of several transcription factors, including, but not limited to, AP-1, NF-κB, and NF-IL6, and the subsequent expression of a multitude of genes [81-83]. The IL-1β signaling cascade begins with the binding of IL-1β to its cognate receptor IL-1RI, followed by the immediate recruitment of the IL-1 receptor accessory protein. This accessory protein acts as a coreceptor and is required for signaling to occur through the nascent receptor complex [81-83]. Upon this complex are recruited several other adapter and activator proteins, such as the IL-1 receptor-associated kinases and myeloid differentiation factor 88 [81, 82, 155]. Similar to TNF-α signaling, the progression of the receptor complex assembly leads to the activation of TAK1 [82]. The exact functions held by TAK1 in IL-1β signaling are still not completely clear. However, TAK1 has been identified as a mediator of IL-1β-induced NF-κB signaling [82]. In addition, one study showed that TAK1 is required for the activation of both the NF-κB and JNK pathways *in vitro* [156], and TAK1 has also been implicated in the upstream events in IL-1β-induced p38 MAPK activation [82]. Furthermore, IL-1β is known to activate all three MAPK pathways, including the ERK pathway as well [81, 83, 155].

As one of the main downstream intracellular effectors of both TNF-α and IL-1β, NF-κB plays a major role in cytokine-induced inflammation. NF-κB is also involved in many other processes and was first discovered as a regulator of the immunoglobulin κ light chain gene in B cells, hence the κB in its name [157, 158]. NF-κB is a family of transcription factors that bind to DNA elements known as κB sites and regulate the expression of over four hundred genes [159]. These include the genes for IL-1β, TNF-α,

CXCL8 [158, 160], CCL2 [112], and CCL20 [113]. NF-κB also regulates adhesion molecules such as intercellular adhesion molecule-1 and vascular cellular adhesion molecule-1 [158, 160], and inflammation-related enzymes such as cyclooxygenase-2, inducible nitric oxide synthase, and matrix metalloproteinases [159]. Also among the genes under the control of NF-κB are several genes with anti-apoptotic activity [159]. These affect the development of dendritic cells, T cells and B cells as well as increasing the survival time of activated neutrophils, which must operate in relatively toxic, pro-apoptotic conditions. In addition, NF-κB induces genes involved in cell growth and in the development of lymphoid organs, which includes regulating homeostatic cytokines that control the proliferation and differentiation of lymphocytes [157, 159].

In mammals, the NF-κB family of transcription factors consists of homodimers and heterodimers of five different gene products: NF-κB1, NFκB2, c-Rel, RelB, and RelA (a.k.a. p65) [as reviewed in 157, 158, 159]. Of the five, both NF-κB1 and NF-κB2 do not contain transactivation domains and thus, must dimerize with one of the remaining three members in order to activate transcription. When unactivated, NF-κB dimers reside in the cytoplasm and are sequestered there by the binding of inhibitory IκB proteins, which eclipse the nuclear localization signals on the NF-κB proteins, preventing their translocation to the nucleus (Figure 1.4). IκB proteins consist of IκBα, IκBβ, IκBγ, IκBε, and Bcl-3 with IκBα being the most abundant. Upon activation of the pathway, IκB proteins are phosphorylated, then ubiquitinated, and rapidly degraded in the proteasome. This leaves NF-κB free to translocate to the nucleus and initiate transcription. Interestingly, one of the transcriptional targets of NF-κB is IκBα itself. This allows for a negative regulatory mechanism in which the degradation of IκBα and release of NF-κB is

followed some time thereafter by a rise in IκBα levels and the subsequent resequestration of NF-κB in the cytoplasm. Alternatively, dimers containing NF-κB2 are not bound by IκB proteins as this protein serves the same purpose as IκB. When activated, the 100 kDa NF-κB2 undergoes ubiquitination- and proteasomal-dependent cleavage to a 52 kDa (p52) form, allowing the p52-containing NF-κB dimer to then translocate to the nucleus.

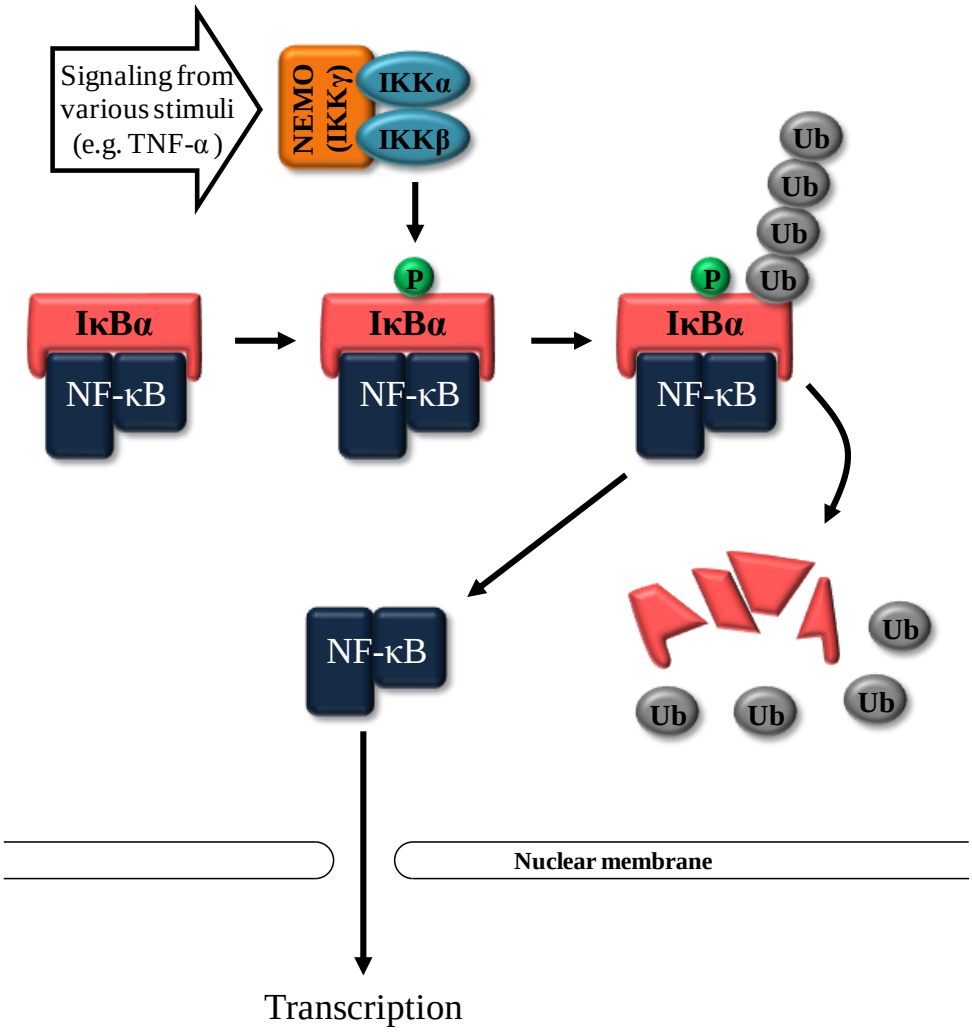

Figure 1.4: The mechanism of NF-κB activation. NF-κB is held in the cytoplasm by the binding of an inhibitory IκB protein, such as IκBα. Upstream signals from a variety of stimuli activate the IKK complex, which then phosphorylates IκBα, marking it for polyubiquitination and subsequent proteasomal degradation. This frees the NF-κB dimer, allowing it to translocate into the nucleus and initiate transcription of a wide array of genes.

Directly upstream of IκB proteins in the NF-κB signaling cascade is the IKK complex (IKK) [as reviewed in 158, 159]. The IKK complex consists of three proteins: IKKα and IKKβ, which are the catalytic subunits, and the regulatory scaffold protein known as NF-κB essential modulator (NEMO a.k.a. IKKγ). The purpose of this complex is to phosphorylate IκB proteins, marking them for degradation. Two mechanisms of NF-κB activation by IKK have been discovered, the canonical and non-canonical pathways. In the canonical pathway, NEMO and IKKβ are required for the phosphorylation of IκB proteins, whereas the non-canonical pathway is dependent on IKKα in the phosphorylation of NF-κB dimers containing the 100 kDa NF-κB2. Upstream, both TAK1 in the canonical pathway and NF-κB-inducing kinase (NIK) in the non-canonical pathway have been identified as activators of IKK, although questions still remain regarding whether these are the only two. Thus, to summarize the canonical pathway, TAK1 activates the IKK complex, which phosphorylates IκB proteins. This leads to the ubiquitination and degradation of IκB proteins and the release of NF-κB, which then translocates to the nucleus and induces various genes. The non-canonical pathway exhibits a similar pattern except that NIK is the upstream activator and it is NF-κB2, instead of a separate IκB protein, which is cleaved to allow for translocation of NF-κB.

Another target of both TNF-α and IL-1β signaling is JNK, a member of the MAPK family of signaling kinases. JNK controls a wide array of cell activities, such as apoptosis, cell differentiation, cell growth, inflammation, and other immune responses [161]. JNK is actually a group of kinases coded by three genes, *JNK1*, *JNK2*, and *JNK3*, each with four splice variants for a total of twelve possible isoforms [162]. *JNK1* and

JNK2 are expressed ubiquitously, while expression of *JNK3* is limited to the heart, brain and testes [163]. Research has shown that each isoform exhibits different substrate affinities [163, 164]. However, the specific roles of each of the different variants of JNK are still poorly understood [161].

The activation of the JNK signaling cascade begins with the activation of a MAP3K by cellular stress or inflammatory stimuli (Figure 1.5) [162]. Several MAP3Ks for JNK have been identified and include MEKK1 - 4, ASK1, ASK2, and possibly TAK1 [162, 163]. Following activation, the MAP3K then phosphorylates one of the two known MAPK kinases (MAP2K) for JNK, MKK4 or MKK7, which in turn, phosphorylate and thus activate JNK [162]. The components of the JNK cascade are organized into signaling cassettes by the binding of a MAP3K, a MAP2K, and JNK to scaffold proteins, which are mainly of the JNK-interacting protein (JIP) family for inflammatory cytokine signaling [162, 165]. There are several JIP proteins, each with its own specific docking sites, allowing for the assembly of specific combinations of signaling components. This allows for control of context-specific responses to various stimuli. In addition to dedicated scaffold proteins, the MAP3K MEKK1 can, itself, act as a scaffold, recruiting and binding MKK4 and JNK to form a signaling cassette [165].

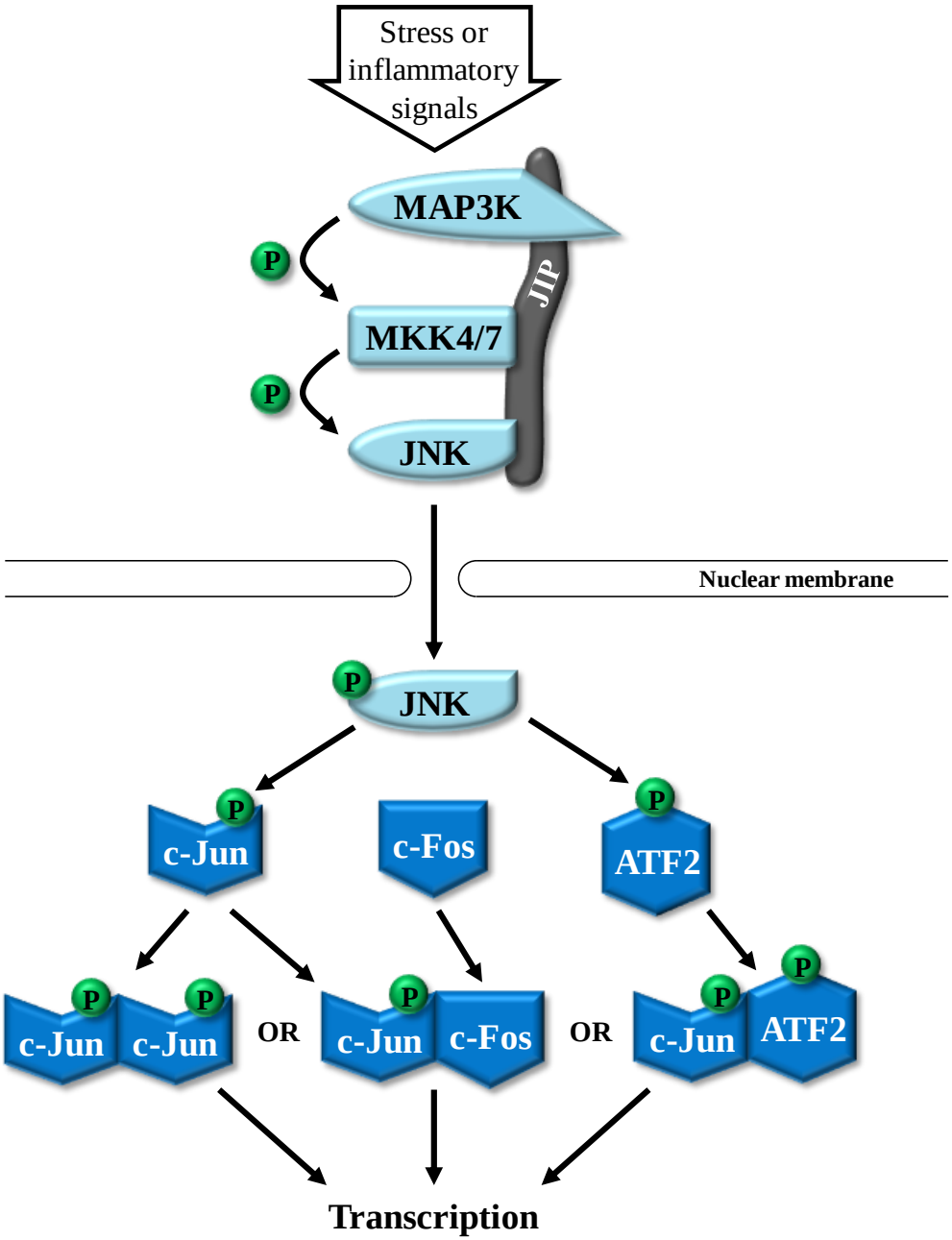

Figure 1.5: JNK and AP-1 signaling. Stress or inflammatory signals initiate signal transduction through the JNK pathway by first activating a MAP3K. There are several kinases known to act as a MAP3K for the JNK pathway, such as MEKK1, ASK1, and possibly TAK1. Once activated, the MAP3K then phosphorylates either MKK4 or MKK7, depending on which has been recruited by the scaffold protein (e.g. JIP). MKK4/7 then phosphorylates JNK, which can subsequently translocate to the nucleus and activate transcription factors such as AP-1. AP-1 consists of hetero- and homodimers of Jun, Fos, and ATF proteins. JNK can phosphorylate Jun and ATF proteins, but not Fos. The resulting activated AP-1 dimers then initiate transcription.

Once activated, JNK can phosphorylate a wide variety of substrates, but perhaps, the most important targets are AP-1 proteins (Figure 1.5). AP-1 is a group of transcription factors consisting of homodimers and heterodimers of Jun (e.g. c-Jun), Fos, and activating transcription factor (ATF) proteins [166]. As its classic substrate, c-Jun is phosphorylated by JNK leading to increased transactivation ability. Phosphorylation of c-Jun by JNK also inhibits its ubiquitination resulting in its stabilization via reduced proteasomal degradation [161]. Similarly, JNK also phosphorylates ATF proteins, enhancing transcriptional activity [163]. Along with AP-1, JNK also activates other transcription factors, such as Elk-1, FOXO4, NFAT, and p53 [161, 163], and thus, regulates the expression of many genes, including the genes for TNF-α, CXCL8, vascular endothelial growth factor, c-Jun, collagenase-3, and aquaporin-1, to name a few [163]. Substrates of JNK also include non-transcription-factor proteins such as the apoptosis-related proteins, Bim, Bad, and BCL-2, as well as the kinases, Akt and $p90^{RSK}$ (ribosomal protein S6 kinase) [161].

The first MAPKs to be discovered were the extracellular signal-regulated kinase 1 (ERK1) and ERK2 [167]. The *ERK1* and *ERK2* genes are highly conserved homologs, and thus, they function and are regulated in a similar manner under most conditions [168]. ERK1/2 are primarily activated by mitogens, like epidermal growth factor (EGF) [168], but also can be activated by stress and inflammatory signals [162]. There are two MAP2Ks that activate ERK1/2: MAPK/ERK kinase 1 (MEK1) and MEK2 [168]. ERK1/2 are the only known substrates of MEK1/2, and it is believed that the interaction of MEK with ERK ensures specificity during the signaling cascade. During mitogenic stimulation of ERK, Raf proteins act as MAP3Ks, phosphorylating MEK, while Ras

proteins act just upstream, activating Raf in the classic Ras-Raf-MEK-ERK cascade [168]. In addition, other MAP3Ks have been identified that are involved in ERK activation during stress or inflammatory signaling, such as MEKK1 [167, 168]. Similar to JNK, which MAP3K is involved in ERK activation is controlled by scaffold proteins, of which there are several for ERK [165]. There are approximately 200 known substrates of ERK [168], which include several transcription factors, such as Elk-1, p53, c-Fos and c-Jun, as well as downstream effector kinases, like $p90^{RSK}$, that further the ERK signal [167]. Through the phosphorylation of its substrates and the subsequent phosphorylation of substrates by effector kinases, ERK regulates a wide variety of cellular processes, for example, proliferation, differentiation, survival, and motility [167].

Rho and Rho-associated coiled-coil containing kinase

The Rho family of proteins belongs to the Ras superfamily of small GTPases [as reviewed in 169, 170]. In humans, there are 23 Rho genes, which are further divided into six subfamilies: Rho, Cdc42, Rac, Rnd, RhoBTB, and RhoT. The Rho subfamily consists of RhoA, RhoB, and RhoC. Like all small GTPases, Rho proteins act as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. When active, Rho GTPases bind to effector molecules causing a conformational change in the effectors, thereby activating them or in a few cases, deactivating them. There are over 70 known Rho effectors, which include many different types of molecules, including scaffold proteins, phospholipases, phosphatases, actin regulatory molecules, and several protein kinases. Through these effectors, Rho proteins regulate many cell activities, such as cell cycle progression, cell morphology and

polarity, vesicle trafficking, microtubule dynamics, actin cytoskeletal rearrangement, acto-myosin contractility, and cell migration through the formation and protrusion of lamellipodia and filopodia. Rho GTPases also alter the expression of genes containing serum response elements in their regulatory regions through the activation of the serum response factor transcription factor [170]. Furthermore, Rho is also known to take part in MAPK signal transduction and can activate both the JNK and p38 pathways [162, 170]. In addition, MEKK1 is known to interact with Rho, raising the possibility that Rho may also regulate ERK signaling as well [170].

An important effector of Rho is the Rho-associated coiled-coil containing protein kinase (ROCK) [as reviewed in 171]. ROCK is a serine/threonine kinase and consists of two evolutionarily conserved homologs with 64% similarity: *ROCK1* and *ROCK2*. Both *ROCK1* and *ROCK2* are expressed nearly ubiquitously in humans and are found together in almost all tissues except the cervix, spleen, thymus, and tongue where *ROCK1* is the sole isoform. The structure of the ROCK protein consists of an N-terminal kinase domain, a subsequent coiled-coil region containing a Rho-binding domain, and an auto-inhibitory region at the C-terminus (Figure 1.6). The auto-inhibitory region interacts with the kinase domain and prevents its activity by an as yet unknown mechanism. When Rho binds to the Rho-binding domain, the auto-inhibitory component is released from the kinase domain, allowing catalytic activity and thus activating ROCK. Activation of ROCK may also occur in a Rho-independent manner through proteolytic cleavage of the auto-inhibitory domain. This occurs during apoptosis when ROCK1 is cleaved by caspase-3 or when ROCK2 is cleaved by the apoptosis-initiating granzyme B, resulting in

constitutively active forms of ROCK and leading to membrane blebbing and formation of apoptotic bodies.

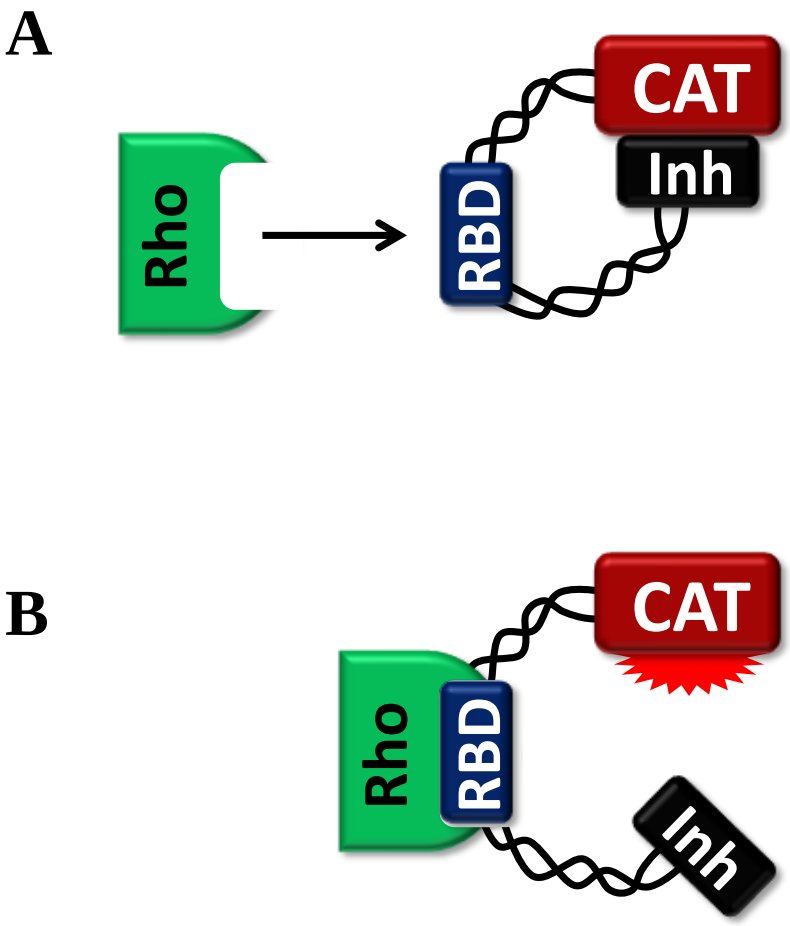

Figure 1.6: A schematic of the structure and activation of ROCK. (A) The structure of ROCK consists of a catalytic domain (CAT), an inhibitory domain (Inh), and an intervening coiled-coil domain that contains a Rho-binding domain (RBD). When ROCK is unactivated, the inhibitory domain binds to the catalytic domain and prevents its activity. **(B)** ROCK is activated by the binding of Rho to the RBD, which causes the inhibitory domain to release from the catalytic domain. (Adapted from Riento and Ridley [172].)

One of the main functions of ROCK is the promotion of acto-myosin contractility [reviewed in 171, 172]. This occurs through regulating the phosphorylation of myosin light chain (MLC), the regulatory subunit of myosin II. ROCK exerts its effect on MLC in two ways: first, by the direct phosphorylation of MLC and second, by the inhibitory phosphorylation of myosin phosphatase target subunit 1, which results in a decreased rate of dephosphorylation of MLC. Both of these activities lead to higher rates of MLC phosphorylation and thus increased contractility. The functional consequences of ROCK-dependent acto-myosin contractility include the formation of focal adhesions and stress fibers, the contraction of the cleavage furrow during cytokinesis, membrane blebbing during apoptosis, and agonist-induced smooth muscle contraction. In addition to attachment to the substratum at focal adhesions, ROCK also is involved in cell-cell adhesion through the modulation of tight junctions. Here the role of ROCK is somewhat ambiguous as ROCK activity can increase or decrease tight junction integrity and paracellular permeability depending on cell type. ROCK has also been implicated in cell migration, and although the details of ROCK's involvement have yet to be fully worked out, it is likely that ROCK promotes contraction at the rear of the cell and regulates the turnover of focal adhesions as the cell moves forward. Furthermore, ROCK is involved in signal transduction leading to the regulation of gene expression as well. For instance, ROCK activity has been shown to attenuate insulin-stimulated phosphorylation of cyclic AMP response element-binding protein, and ROCK has also been found to mediate lysophosphatidic acid (LPA)-induced activation of c-Jun [173].

In a recent study, Segain *et al.* [123] found a link between ROCK and intestinal inflammation. In one set of experiments, the Segain group found that levels of active,

GTP-bound RhoA were significantly elevated in inflamed tissues from biopsies from CD patients compared to non-inflamed tissues from CD patients and compared to control patients. The same pattern of RhoA activation was also observed in trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats. Shifting the focus to ROCK, they also found that treatment with Y-27632, a specific inhibitor of ROCK, significantly reduced the amount of TNF-α spontaneously secreted by lamina propria mononuclear cells isolated from both inflamed tissues of CD patients and from TNBS-treated rats, indicating that ROCK mediates TNF-α production in these cells. In addition, TNBS-treated rats that were administered the ROCK inhibitor also scored better on several histological and physiological measures of inflammation than did control rats, pointing toward the involvement of ROCK in overall inflammatory processes in the intestine. Similar to what was seen with mononuclear cells isolated from inflamed tissues, LPS treatment of peripheral blood mononuclear cells induced RhoA activation and the production of both TNF-α and IL-1β, and furthermore, TNF-α and IL-1β production was significantly abrogated by the inhibition of ROCK. ROCK inhibition was also found to prevent the activation of the NF-κB pathway. In that set of experiments, Segain *et al.* showed that in peripheral blood mononuclear cells, treatment with either LPS or TNF-α stimulated the degradation of IκBα and the translocation of NF-κB to the nucleus, and both of these effects were blocked by treatment with the ROCK inhibitor, which suggests that ROCK is required for the activation of NF-κB in these cells. These results, taken together suggest that ROCK may play a critical role in intestinal inflammation and that the inflammatory signaling activities of ROCK are mediated by NF-κB. To note, Segain

et al. focused on inflammation in mononuclear cells and whole tissue samples and did not examine IEC, which compose a major component of the mucosal immune system.

ROCK has been linked to inflammatory signaling in other contexts as well. For example, inhibiting ROCK was shown to block the secretion of CXCL8, CCL5, and CCL8 in peptidoglycan-treated synoviocytes from rheumatoid arthritis patients [174]. ROCK inhibition was also shown to significantly reduce CXCL8 secretion in LPS-stimulated cervical stromal cells, and this effect was mediated by NF-κB [175]. Clearly, ROCK plays a role in inflammatory signaling. However, there is still much to be learned regarding its cell-specific effects, its stimulus-specific effects, and which signaling pathways are under its regulatory purview.

Recently, experiments in our laboratory have confirmed a role for ROCK in inflammatory responses in IEC [176]. When ROCK was inhibited with Y-27632 in the Caco-2 IEC cell line, there was a significant reduction in IL-1β-induced CXCL8 secretion and mRNA expression as compared to IL-1β-only controls. Likewise, there was a significant reduction in IL-1β-induced CCL2 secretion and mRNA expression in IEC-6 cells when ROCK was inhibited. Together, these results suggest that ROCK may be an important mediator of CXCL8, CCL2, and possibly other chemokines in IEC. In contrast to the results found by the Segain group [123], the experiments in our laboratory showed that, in Caco-2 cells, ROCK inhibition had no effect on IL-1β-stimulated NF-κB activation as the rates of IκBα phosphorylation and degradation were unchanged by ROCK inhibition and there was no effect on IL-1β-stimulated IKK activation. ROCK inhibition did have an effect on JNK signaling, however. Inhibiting ROCK in Caco-2 cells and in IEC-6 cells led to a suppression of JNK signaling as shown by a significant

decrease in IL-1β-induced phosphorylation of p46 JNK and p54 JNK, indicating that JNK signaling may be under the control of ROCK in IEC. This is in accord with another study that found that the activation of JNK was mediated by ROCK in the NIH 3T3 mouse embryonic fibroblast cell line, albeit not in response to IL-1β [173]. Taken together, these results suggest that ROCK may play an important part in inflammatory signal transduction in IEC and possibly other cell types.

Still, more research is needed if we are to understand IBD, the role that IEC play in IBD, and the role that ROCK may play in the persistent inflammatory signals that characterize IBD. From our previous work, we know that ROCK is involved in IL-1β-induced CXCL8 and CCL2 responses in IEC and in IL-1β signaling through JNK. However, little is known about the role of ROCK in the regulation of other chemokines or the role of ROCK in the transduction of inflammatory signals other than IL-1β. Since TNF-α plays a central role in IBD and since IEC are a large component of the mucosal immune system, we turned our focus to examining the involvement of ROCK in TNF-α-induced responses in IEC. This included investigating the involvement of ROCK in the production of CXCL8, CCL2, and CCL20, as well as examining the effects of ROCK in several signaling pathways. Understanding the role of ROCK in IEC may open up new avenues of research into IBD and may identify ROCK as a potential therapeutic target for the reduction of inflammation in IBD. A ROCK inhibitor drug could be used alone or as a complement to offset the shortcomings of current treatments for those who do not respond well.

Chapter 2

The Role of the ROCK Kinase in Tumor Necrosis Factor-α-Induced CXCL8 Secretion in Intestinal Epithelial Cells

1. Introduction

TNF-α is a major pro-inflammatory cytokine that plays a central role in Inflammatory Bowel Disease (IBD) [177]. Elevated levels of TNF-α have been detected in the serum [119] and stool [117] of patients affected with IBD, along with a corresponding increase in the production of TNF-α by cells in the intestinal lamina propria [78, 120, 121] and the intestinal epithelium [178]. As the frontline defense against pathogens, the cells of the intestinal epithelium are in constant and intimate contact with the intestinal contents, and since they are the first to encounter luminal antigens, IEC likely participate in the inappropriate immune response seen in IBD. Indeed, increased intestinal epithelial permeability has been implicated in the development of IBD [179-183], and IEC have been shown to produce several cytokines and other molecules involved in the progression and maintenance of the inflammatory response [48, 51, 63, 184-186]. Because of the central role of TNF-α in IBD, it is not surprising that TNF-α can modulate IEC responses. Accordingly, TNF-α has been found to stimulate the secretion of CXCL1, CXCL3, CXCL5, CXCL8, CXCL10, CCL2, CCL4, CCL5, CCL20, granulocyte/monocyte colony stimulating factor, IL-1β, IL-6, as well as

TNF-α itself by several IEC cell lines [49-51, 97, 114, 150, 151]. While IEC responses to TNF-α are fairly well characterized, more research is needed to elucidate the intracellular mechanisms that mediate the actions of TNF-α if new treatments for IBD are to be devised.

Transduction of the TNF-α signal is highly complex and entails simultaneous actuation of multiple signaling pathways. Despite this complexity, the regulation of TNF-α-stimulated gene expression is mainly achieved through the activation of two transcription factors: NF-κB and AP-1 [187]. NF-κB is ubiquitously expressed and is involved in a wide array of cellular activities, including growth, survival, differentiation, cell adhesion, and inflammatory response. In its unactivated state, the NF-κB transcription factor is bound by inhibitory IκB proteins, most often IκBα, and is sequestered in the cytoplasm. An essential step in the activation of NF-κB is the phosphorylation and subsequent proteasomal degradation of IκBα. Upon phosphorylation and degradation, IκBα releases its binding to NF-κB, allowing the translocation of NF-κB into the nuclear compartment [158, 160]. Accordingly, studies with IEC cell lines have demonstrated the TNF-α-induced activation of NF-κB by showing that IκBα is rapidly phosphorylated and degraded upon TNF-α stimulation and by detecting increased nuclear localization and promoter binding activity of NF-κB [188-191]. Once translocated to the nucleus, NF-κB binds to DNA at κB regulatory sites and activates the transcription. Among the many TNF-α-induced and inflammation-related genes that are controlled by NF-κB are IL-1β, TNF-α itself, and several chemokines such as CXCL1, CXCL8, CCL2, and CCL5 [158, 160].

The AP-1 family of transcription factors is comprised of hetero- and homodimeric proteins consisting of combinations of Jun, Fos, and ATF proteins, with the c-Jun/c-Fos form being the prototypical member of this family [192]. AP-1 members play varied roles in apoptosis, cell survival, differentiation, proliferation, and inflammation [193-195]. An important regulator of AP-1 is JNK, which activates AP-1 by phosphorylating Jun or ATF proteins in response to many different stimuli, including TNF-α [187, 196]. JNK proteins are coded by three genes, two of which, *JNK1* and *JNK2*, are expressed ubiquitously [196]. In addition, alternative splicing of JNK genes can yield isoforms of approximately 46 and 54 kDa, which confers differences in substrate specificity and provides a mechanism for the selective activation of subsets of AP-1 proteins [164]. In inflammation, AP-1 activates the transcription of TNF-α, IL-1, IL-6, CXCL8, and CCL2, among others [107, 194, 195, 197-202].

TNF-α has also been shown to activate p38 MAPK, another signaling kinase that can modulate AP-1- and NF-κB-directed transcription [187]. For instance, p38 MAPK has been shown in some cases to phosphorylate ATF2 thereby activating AP-1 [192, 203], while a combination of p38 MAPK and ERK activity has been implicated in regulating histone acetylation required for NF-κB-dependent transcription in mouse fibrosarcoma cells [204]. The p38 MAPK also activates Elk-1, a transcription factor involved in initiating the expression of the *c-fos* gene [203]. In addition to regulating transcription, p38 MAPK also affects the expression of many pro-inflammatory genes by regulating mRNA decay through the phosphorylation of AU-rich element (ARE)-binding proteins [162, 205, 206]. These ARE-binding proteins then bind to AREs found in the

non-translating 3' regions of mRNAs and modulate the rate of deadenylation-dependent mRNA decay.

As the focus of research for many years, the basics of TNF-α signaling are well established. However, many details regarding the mechanisms of TNF-α-induced gene expression are still unknown. Particularly which variations in signal transduction exist across cell types and conditions. This includes the specifics of the regulation of NF-κB- and AP-1-mediated gene expression as well as signaling through p38 MAPK and other kinases.

One potential mediator of TNF-α signaling is the Rho-associated coiled-coil kinase (ROCK). ROCK is a serine/threonine kinase and exists in two isoforms, ROCK1 and ROCK2, which are coded by two highly homologous genes [172]. ROCK is activated by the binding of Rho family GTPases and is often associated with cellular processes that involve acto-myosin contractility, cytoskeletal rearrangement, and cell adhesion, including motility, stress fiber formation, and membrane blebbing during apoptosis [170, 172]. However, a recent study by Segain *et al.* [123] found a link between ROCK and intestinal inflammation. In that study, inhibition of ROCK reduced intestinal inflammation in rats with experimentally induced colitis and suppressed the production of TNF-α by lamina propria mononuclear cells isolated from the treated rats as well as from patients with CD. Additionally, blockade of ROCK with the chemical inhibitor Y-27632 prevented TNF-α-induced activation of NF-κB in peripheral blood mononuclear cells isolated from active-CD tissues. Nwariaku *et al.* [207] also found a link between ROCK and inflammation when they showed that inhibiting Rho/ROCK signaling reduced p38 MAPK activity and abolished ERK activity stimulated by TNF-α

in human lung microvascular endothelial cells. Taken together, these results suggest that ROCK may be an important mediator of TNF-α-induced inflammation and that inhibiting ROCK or its downstream targets may be an effective means for alleviating inflammation in IBD that merits further research.

Of particular interest is what role ROCK may play in modulating TNF-α responses in IEC. The full extent of IEC participation in IBD etiology is unclear. However, IEC are known to react to inflammatory stimuli by secreting relatively large amounts of the neutrophil chemoattractant CXCL8 [49, 51], a chemokine found at elevated levels in IBD [74, 144]. In this way, IEC may contribute to the beginning stages of IBD pathology by initiating leukocyte influx or may provide a continuous signal that maintains chronic inflammation. In synoviocytes [174], human cervical stromal cells [175], and human umbilical vein endothelial cells (HUVECs) [208, 209], ROCK has been shown to play a part in the regulation of CXCL8 secretion, albeit in response to stimuli other than TNF-α. Additionally, a recent study in our laboratory showed that the inhibition of ROCK in an IEC cell line led to the suppression IL-1β-induced CXCL8 secretion and mRNA expression and that this corresponded to decreased JNK, but not NF-κB signaling [176]. Here we report the results of the first investigation of the function of ROCK in IEC responses to TNF-α. Similar to our previous findings with IL-1β, inhibiting ROCK resulted in a significant reduction in the TNF-α-induced secretion and mRNA expression of CXCL8. Furthermore, our results show a concomitant suppression of TNF-α-induced JNK activation, while ROCK inhibition had no effect on the TNF-α-stimulated activation of the NF-κB and p38 MAPK pathways.

2. Materials and Methods

2.1 Antibodies.

All antibodies used in this study were obtained from Cell Signaling Technologies (Beverly, MA). These included rabbit polyclonal antibodies against JNK (recognizing both p46 and p54 isoforms), p38 MAPK, and β-actin; rabbit monoclonal antibodies against phosphorylated p38 MAPK (Thr180/Tyr182) and phosphorylated IκBα (Ser32); and mouse monoclonal antibodies against phosphorylated JNK (Thr183/Tyr185) and IκBα. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies (Cell Signaling Technologies) were used for detection.

2.2 Cell culture conditions.

The Caco-2 human colonic cell line was obtained from the American Type Culture Collection (ATCC HTB-37; Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Logan, UT) containing 10% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY), 3.7 g/L sodium bicarbonate, 2 mM L-glutamine (Lonza, Walkersville, MD), non-essential amino acids (Lonza), and 25 IU penicillin with 25 μg/mL streptomycin (Mediatech, Herndon, VA). This medium is subsequently referred to as FBS-DMEM. Cultures were maintained at 37 °C in a 10% CO_2 humid atmosphere.

Caco-2 cells were cultured in 6-, 12-, or 24-well tissue culture plates coated with fibronectin (FN). To coat culture plates, 20 μg/mL bovine plasma FN (Sigma-Aldrich, St. Louis, MO) in PBS was added to wells at 40 μL/cm^2 according to the

growth area for each well size and incubated for 1 h at 37 °C. The fluid was then aspirated, and the wells were allowed to dry in a biosafety cabinet under UV illumination before storage at 4 °C until needed. To seed the plates, cells were removed from culture flasks by treatment with a trypsin/EDTA solution (Sigma-Aldrich) and centrifuged for 5 – 10 min. The cell pellets were then resuspended in FBS-DMEM, added to wells at the required densities, and incubated for 1 h at 37 °C to allow for attachment. After this, the medium was removed, and the cells were treated with or without 40 μM of the Y-27632 ROCK inhibitor (Enzo Life Sciences, Farmingdale, NY) in serum-free DMEM with an additive premix of insulin, transferrin, and selenium (ITS; BD Biosciences, Bedford, MA) and incubated for 2 h. This was followed by treatment with either recombinant human (rh)TNF-α (R&D Systems, Minneapolis, MN) at a concentration of 10 ng/mL or rhIL-1β (R&D Systems) at a concentration of 1 ng/mL and further culturing as indicated below. Prior work in our laboratory has shown these concentrations of IL-β and TNF-α to be optimal for the study of cytokine responses in Caco-2 cells [97].

2.3 Preparation of RNA extracts.

Total RNA was isolated from Caco-2 cells using the Qiagen (Valencia, CA) RNeasy kit according to the manufacturer's protocol. Caco-2 cells were added to 6-well plates (1×10^6 cells/well) and treated with Y-27632 as described above. After 2 h incubation, the cells were treated with either rhTNF-α or rhIL-1β and incubated for an additional 6 h. At the end of the incubation period, each well was aspirated to remove the medium, and the cellular contents were extracted using the lysis buffer supplied in the RNeasy kit. Lysates were homogenized by several passes through 20- or 21-gauge hypodermic

needles, and RNA was isolated by transferring the lysates to RNeasy spin columns and then centrifuging. The columns were then washed with the supplied wash solutions, and RNA was eluted in RNase-free water. Sample concentration and purity was determined by measuring A_{260}, A_{280}, and A_{230} with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE).

2.4 Quantification of relative CXCL8 mRNA levels.

Relative mRNA levels were determined using real-time RT-PCR. First, reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Briefly, 1 µg of total RNA from each sample was converted to cDNA in a 20 µL reaction containing 10 µL of RNA sample diluted to 0.1 µg/mL in nuclease-free water, an additional 3.2 µL of nuclease-free water, 2 µL of the supplied RT buffer, 0.8 µL of dNTP mix (4 mM final concentration), 2 µL of the supplied mix of random hexamer primers, 1 µL of RNase inhibitor, and 1 µL of MultiScribe reverse transcriptase (50 U /reaction). Reactions were incubated in a thermocycler for 10 min at 25 °C to allow for primer annealing, then for 2h at 37 °C for the reverse transcription reaction, followed by 5 min at 85 °C to denature the enzyme.

The resulting cDNA samples were then analyzed by real-time PCR. Each 20 µL reaction contained 2 µL of cDNA sample, 10 µL of iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA), 0.8 µL of 10 µM forward/reverse primer mix, and 7.2 µL RNase-free water. Human CXCL8 and human GAPDH primer mixes were purchased from SABiosciences/Qiagen (Valencia, CA). Real-time PCR was performed using a

BioRad MiniOpticon thermocycler running the following program: 95 °C for 3 min, then 40 two-step cycles of 95 °C for 15 s and 60 °C for 60 s, followed by a melting curve determination. Relative transcript levels were calculated with the $2^{-\Delta\Delta Ct}$ method using GAPDH as the reference gene, and single-product amplification was verified by examination of the PCR product melt curves.

2.5 Immunoblot analysis.

In order to determine levels of intracellular proteins, immunoblot analysis was performed on cytoplasmic extracts as follows. Cells were seeded in 12-well plates at a density of 1×10^6 cells/well, treated with the ROCK inhibitor as indicated above, and then incubated for 0, 15, 30, or 45 min with or without 10 ng/mL rhTNF-α. The cells were then washed with cold PBS and scraped from culture plates using plastic cell scrapers in the presence of 100 µL/well of lysis buffer containing 10 mM Tris-HCl, 5 mM disodium EDTA, 60 mM potassium chloride, 0.4% IGEPAL CA-630, 20 mM 4-nitrophenylphosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 40 mM β-glycerophosphate, 20 mM sodium fluoride, and 1.44 µL/mL of Protease Inhibitor Cocktail Set III (100 mM AEBSF, 80 µM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, and 1 mM pepstatin A; Calbiochem, San Diego, CA). The cell/buffer suspensions were then transferred to microcentrifuge tubes and incubated on ice for 10 min to allow for cell lysis. The resulting lysates were centrifuged at 12,000 rpm for 10 minutes at 4 °C to remove nuclei and any debris. Protein concentrations of the lysates were determined with the Bio-Rad DC protein assay kit.

Protein samples were diluted to equal concentrations with lysis buffer as needed, and then three parts of the resulting protein dilutions were mixed with one part Laemmli buffer containing 8% sodium dodecyl sulfate, 40% glycerol, 0.08% bromphenol blue, and 10% β-mercaptoethanol in 0.4 mM Tris-HCl buffer (6.8 pH). These mixtures were heated to 95 °C for 5 min, and separated by SDS-PAGE on 10% polyacrylamide gels with 4% stacking gels. Proteins were then transferred to polyvinylidene difluoride membranes with the BioRad Mini Trans-blot system. Transfers were run at 100 V for 1 h with ice cooling. For blocking, the membranes were incubated in 5% fraction V bovine serum albumin (BSA; Fisher Scientific, Fair Lawn, NJ) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 h at room temperature. To probe for specific proteins, the membranes were incubated with primary antibody in 5% BSA/TBST overnight at 4 °C with constant rocking. The membranes were then washed 3 times with TBST for 5 min per wash and incubated at room temperature with the appropriate HRP-conjugated secondary antibody in 2.5% BSA/TBST for 2 h. The blots were then washed another 3 times with TBST before protein bands were detected using either LumiGLO Reagent (Cell Signaling Technologies) or SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) detection systems. Images were captured by exposure on x-ray film. For reprobing, membranes were washed twice in TBST, incubated at 50 °C for 12 min in stripping buffer containing 2% SDS, 100 mM 2-mercaptoethanol, and 62.5 mM Tris-HCl, and washed an additional 3 times in TBST before being reprocessed starting at the blocking step described above. Densitometry was performed by scanning films and analyzing images using the BioRad Quantity One software.

2.6 Determination of CXCL8 levels in culture supernatants.

Caco-2 cells cultured in 24-well plates (2×10^5 cells/well) were treated with the ROCK inhibitor for 2 h as previously described and incubated for 24 h in the presence of rhTNF-α (10 ng/mL) or rhIL-1β (1 ng/mL). Culture supernatants were then collected and analyzed for CXCL8 content using a DuoSet ELISA kit (R&D Systems) according to the manufacturer's protocol. Immediately after supernatant collection, trypsin/EDTA was added to each well, and the number of cells per well was determined by direct count using a hemacytometer. Color development in the ELISA plates was quantified using a Bio-Tek EL312e microplate reader (Bio-Tek Instruments Inc., Winooski, VT), and the resulting CXCL8 concentration values were normalized to 10^5 cells/well. An analysis of cell counts showed that the ROCK inhibitor had no effect on cell viability ($p = 0.98$, data not shown).

2.7 Statistics.

All experiments were run in three or more replicates ($n \geq 3$), and the statistical significance of any observed differences in treatment effects was determined using ANOVA with Fisher's protected least significant difference post-hoc test. Differences with $p < 0.05$ are reported as significant.

3. Results

3.1 *The effect of inhibiting ROCK on TNF-α- and IL-1β-induced CXCL8 secretion in Caco-2 cells*

TNF-α and IL-1β are cytokines that are secreted by many cells types and play major roles in inflammation. Not surprisingly, both of these cytokines have been found at elevated levels in the intestinal tissues of IBD patients, suggesting that both may be important in the development and progression of this disease [117, 120, 125, 132]. In addition to TNF-α and IL-1β, several other inflammatory mediators have also been found at elevated levels in IBD, including several chemokines, and in particular, CXCL8 [74, 144]. CXCL8 is known to be secreted by IEC [49] and is a major chemoattractant for neutrophils, which further the progression of inflammation and produce substances responsible for tissue damage in prolonged inflammation [45]. Previous experiments in our laboratory have indicated that the signaling kinase, ROCK, plays a role in the regulation of CXCL8 and other chemokine secretion in IEC cell lines stimulated with IL-1β [176]. Because TNF-α is likewise elevated in IBD and is a major target in IBD treatment, we asked whether the regulatory mechanism of TNF-α-induced CXCL8 secretion may also involve ROCK.

In a previous study, the effect of Y-27632, an inhibitor that has a high specificity for ROCK [210-212], on IL-1β-induced CXCL8 secretion was examined by treating Caco-2 cells with inhibitor concentrations ranging from 1 μM to 100 μM at 10-fold intervals [176]. As would be expected, the strongest suppression of IL-1β-induced CXCL8 secretion occurred at an inhibitor concentration of 100 μM, while the next highest concentration (10 μM) did not produce a significant suppression at all. Although

Y-27632 concentrations of 100 μM or more have been used in many studies [211, 213-216], we reduced the dose to 40 μM in these current experiments in order to avoid any potential off-target inhibitions that can occur at higher doses [211, 212]. In order to confirm that a lower dose would still produce a significant suppression of IL-1β-induced CXCL8 secretion, the previous experiments were repeated using 40 μM Y-27632. As shown in Figure 2.1B, when the Caco-2 cells were treated with the 40 μM dose of the ROCK inhibitor along with IL-1β, there was a significant 69% decrease in CXCL8 production compared to cells treated with IL-1β alone ($p < 0.01$). Furthermore, the lower dose of ROCK inhibitor produced the same decrease (reported as 70%) observed with the 100 μM concentration in the previous experiments [176]. This strongly suggests that the observed effect of the ROCK inhibitor is due to the direct suppression of ROCK activity and is not a side effect of an off-target inhibition that would likely not be observed at the lower dose. Additionally, the results of later dose-response experiments (Chapter 3, Figure 3-5) showed that, while 10 μM ROCK inhibitor had a significant effect in TNF-α-treated Caco-2 cells, the peak of the dose-dependent response occurred near 40 μM, providing further support for the use of the 40 μM concentration.

We next investigated the role of ROCK in TNF-α-stimulated CXCL8 secretion. Caco-2 cells were seeded in FN-coated wells and incubated for 1 h to allow for attachment, followed by treatment with 40 μM Y-27632. After an additional 2 h, the cells were treated with or without TNF-α and incubated for 24 h. Analysis of the resulting culture supernatants showed that levels of CXCL8 secreted by the Caco-2 cells treated with the ROCK inhibitor were similar to levels secreted by untreated cells. However, cells treated with TNF-α in addition to the ROCK inhibitor produced a

significant 46% less CXCL8 ($p < 0.05$) than cells treated with TNF-α alone (Figure 2.1A). This observed reduction of CXCL8 secretion with ROCK inhibitor treatment suggests that ROCK may play an important role in regulating TNF-α-stimulated CXCL8 production in these cells.

3.2 *CXCL8 mRNA levels were also suppressed by the ROCK inhibitor in TNF-α- and IL-1β-treated Caco-2 cells*

To determine if the ROCK inhibitor was suppressing CXCL8 secretion by regulating mRNA levels, Caco-2 cells were again plated in FN-coated wells and treated with or without the ROCK inhibitor and TNF-α as previously described. After a 6 h incubation, total RNA was isolated from the cells, and relative CXCL8 transcript levels were determined by real-time RT-PCR. Similar to its effect on secretion, the ROCK inhibitor also had a significant suppressive effect on TNF-α-induced CXCL8 mRNA levels, reducing it by 82% from levels in cells treated with TNF-α alone (Figure 2.2A). Additionally, as with IL-1β-induced CXCL8 secretion, previous experiments in our laboratory used a concentration of 100 μM of ROCK inhibitor to examine its effect on mRNA levels in IL-1β-treated Caco-2 cells. Thus, the efficacy of using 40 μM ROCK inhibitor was again tested. CXCL8 transcript levels in Caco-2 cells treated with both 40 μM ROCK inhibitor and IL-1β were significantly decreased by 57% compared to cells treated with IL-1β alone, confirming that the 40 μM dose is sufficiently effective. Furthermore, in both experiments, the ROCK inhibitor alone had no significant effect on basal levels of CXCL8 mRNA.

3.3 TNF-α-induced phosphorylation of p38 MAPK was unaffected by the ROCK inhibitor.

The activation of p38 MAPK has been shown to be an important regulation event in CXCL8 secretion in some cell types. In particular, Zu et al. [217] found that p38 MAPK activation is necessary for TNF-α-induced CXCL8 secretion in isolated human neutrophils, while Maresca et al. [218] demonstrated a similar role for p38 MAPK in mycotoxin-induced CXCL8 secretion in Caco-2 cells. Additionally, a study by Nwariaku et al. [207] showed that the blockade of ROCK with Y-27632 abrogates p38 MAPK kinase activity in human lung microvascular endothelial cells. To investigate if ROCK may affect the activation of p38 MAPK in our system, we next examined the effect of ROCK inhibitor on p38 MAPK phosphorylation in TNF-α-stimulated cells. Again, Caco-2 cells were plated and pre-treated with the ROCK inhibitor as described in the Materials and Methods. After the addition of TNF-α, the cells were incubated for 0, 15, 30, and 45 min and then lysed. To determine levels of p38 MAPK phosphorylation, the resulting cytoplasmic extracts were analyzed by Western blot (Figure 2.3). After 15 min, there was a significant 9-fold increase in p38 MAPK phosphorylation in cells treated with TNF-α alone ($p < 0.01$), which was sustained at similar levels through 45 min of the experiment. In contrast to the previous results, however, the ROCK inhibitor had little to no effect on the phosphorylation of p38 MAPK at any time point, indicating that inhibiting ROCK does not affect the activation of this kinase in Caco-2 cells.

3.4 Inhibiting ROCK had little to no effect on IκBα phosphorylation and degradation.

The NF-κB pathway plays a major role in inflammatory signal transduction. An important event in the activation of this pathway is the phosphorylation and subsequent proteasomal degradation of IκBα, which normally binds to NF-κB and prevents its translocation to the nucleus. To determine whether the NF-κB pathway played a role in the observed effect of the ROCK inhibitor on CXCL8 secretion in Caco-2 cells, Western blot was utilized to examine both the phosphorylation and the degradation of IκBα. As shown in Figure 2.4A, in cells stimulated with TNF-α alone, the level of IκBα phosphorylation greatly increased by the 15 min mark, resulting in a 16-fold increase in the ratio of phosphorylated to total IκBα compared to that of the unstimulated controls (Figure 2.4B). As expected, this finding was matched by an apparent reduction in the overall IκBα levels in those same cells (Figure 2.4C). However, this reduction was not statistically significant. In cells treated with the ROCK inhibitor prior to TNF-α addition, a similar increase in IκBα phosphorylation and decrease in total IκBα occurred, neither of which were significantly different from the patterns seen with TNF-α alone. While this evidence suggests that the NF-κB pathway is being activated by TNF-α, the inability of ROCK inhibition to suppress IκBα phosphorylation and its subsequent degradation suggests that ROCK does not participate in NF-κB activation under the conditions of these experiments.

3.5 The effect of inhibiting ROCK on JNK activation in TNF-α-treated Caco-2 cells.

TNF-α and IL-1β are both known to activate JNK, an important regulator of inflammation-related genes. JNK is expressed as p46 and p54 isoforms, each having

different substrate specificities, and acts mainly by phosphorylating of members of the Jun family of proteins [164]. Classically, Jun proteins are known to form dimers with Fos proteins, which, together, make up the AP-1 transcription factor [166, 192]. Because the CXCL8 promoter contains an AP-1 binding site [107], we next turned our attention to the effect of inhibiting ROCK on TNF-α-induced JNK activation. As shown in Figure 2.5, treating the Caco-2 cells with TNF-α had no effect on the levels of phosphorylated p46 JNK over the course of the 45 min time period, with or without the ROCK inhibitor. This suggests that TNF-α does not activate p46 JNK in Caco-2 cells. However, there was a significant 4.7-fold induction of p54 JNK phosphorylation by 15 min after the addition of TNF-α. Furthermore, this TNF-α-induced elevation in p54 JNK phosphorylation was reduced 50% by treatment with the ROCK inhibitor. This reduction of TNF-α-induced activation of p54 JNK suggests a mechanism by which the ROCK inhibitor suppresses CXCL8 secretion in TNF-α-stimulated Caco-2 cells.

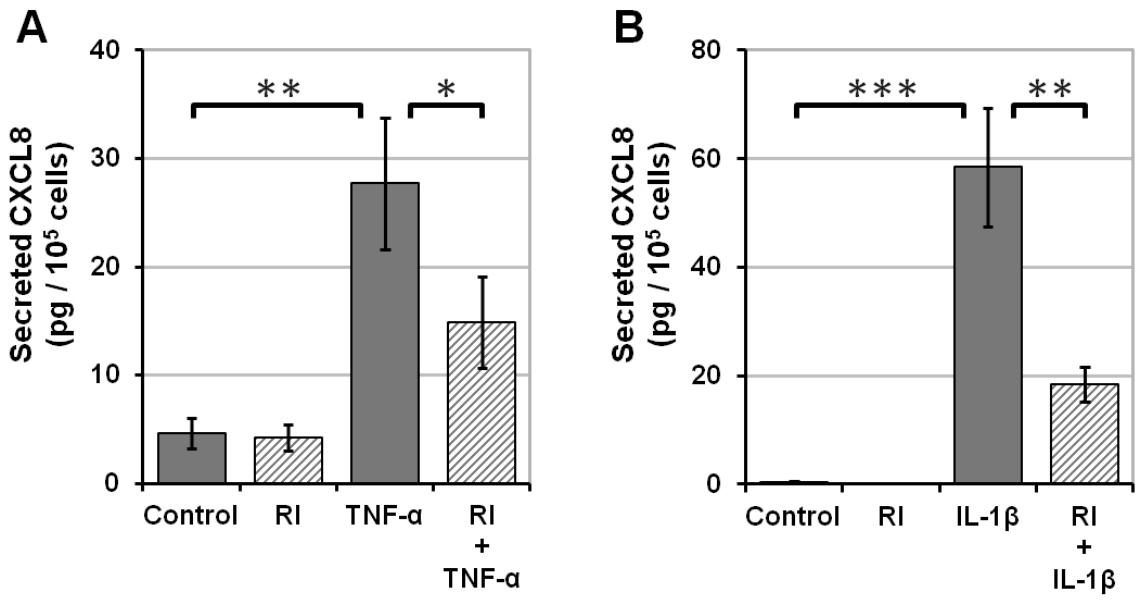

Figure 2.1: The Y-27632 ROCK inhibitor suppresses CXCL8 secretion in Caco-2 cells treated with either TNF-α or IL-1β. Caco-2 cells suspended in FBS-DMEM were plated in FN-coated 24-well culture plates and allowed to attach for 1 h at 37 °C. The medium was then removed and replaced with ITS-DMEM with or without 40 µM Y-27632 ROCK inhibitor (RI). The cells were then incubated for 2 h before the addition of either **(A)** TNF-α to a final concentration of 10 ng/mL or **(B)** IL-1β to a final concentration of 1 ng/mL. After an additional 24 h incubation period, culture supernatants were collected, and the cells in each well were counted. Supernatants were analyzed for secreted CXCL8 by ELISA, and the resulting concentrations were normalized to cell counts. Bars represent averages of 3 replicates ± 1 SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

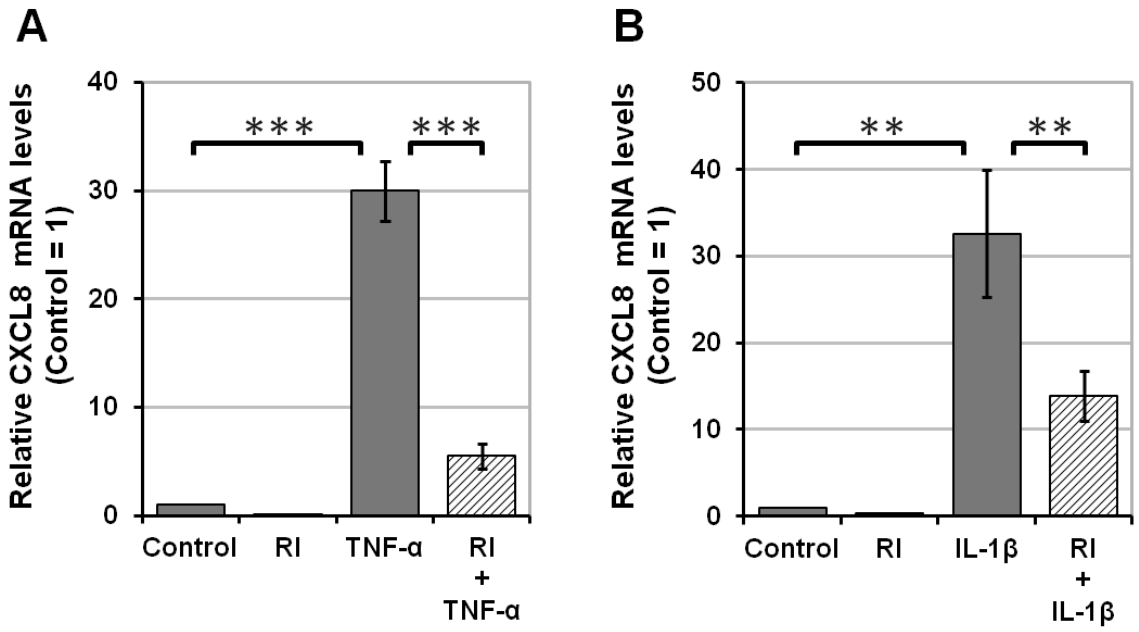

Figure 2.2: The effect of inhibiting ROCK on TNF-α- and IL-1β-induced increases in CXCL8 mRNA expression in Caco-2 cells. Caco-2 cells were seeded in FN-coated 6-well culture plates (1×10^6 cells/well) and pre-treated with ROCK inhibitor as described in the Materials and Methods. Cells were then treated with **(A)** TNF-α (10 ng/mL) or **(B)** IL-1β (1 ng/mL) and allowed to incubate for 6 h. Total RNA was then extracted from the cells and analyzed using reverse transcription real-time PCR. CXCL8 transcript levels were normalized to GAPDH transcript levels and expressed as a value relative to unstimulated controls. Means ± 1 SEM of (A) 3 and (B) 4 independent experiments are shown. (** $p < 0.01$, *** $p < 0.0001$).

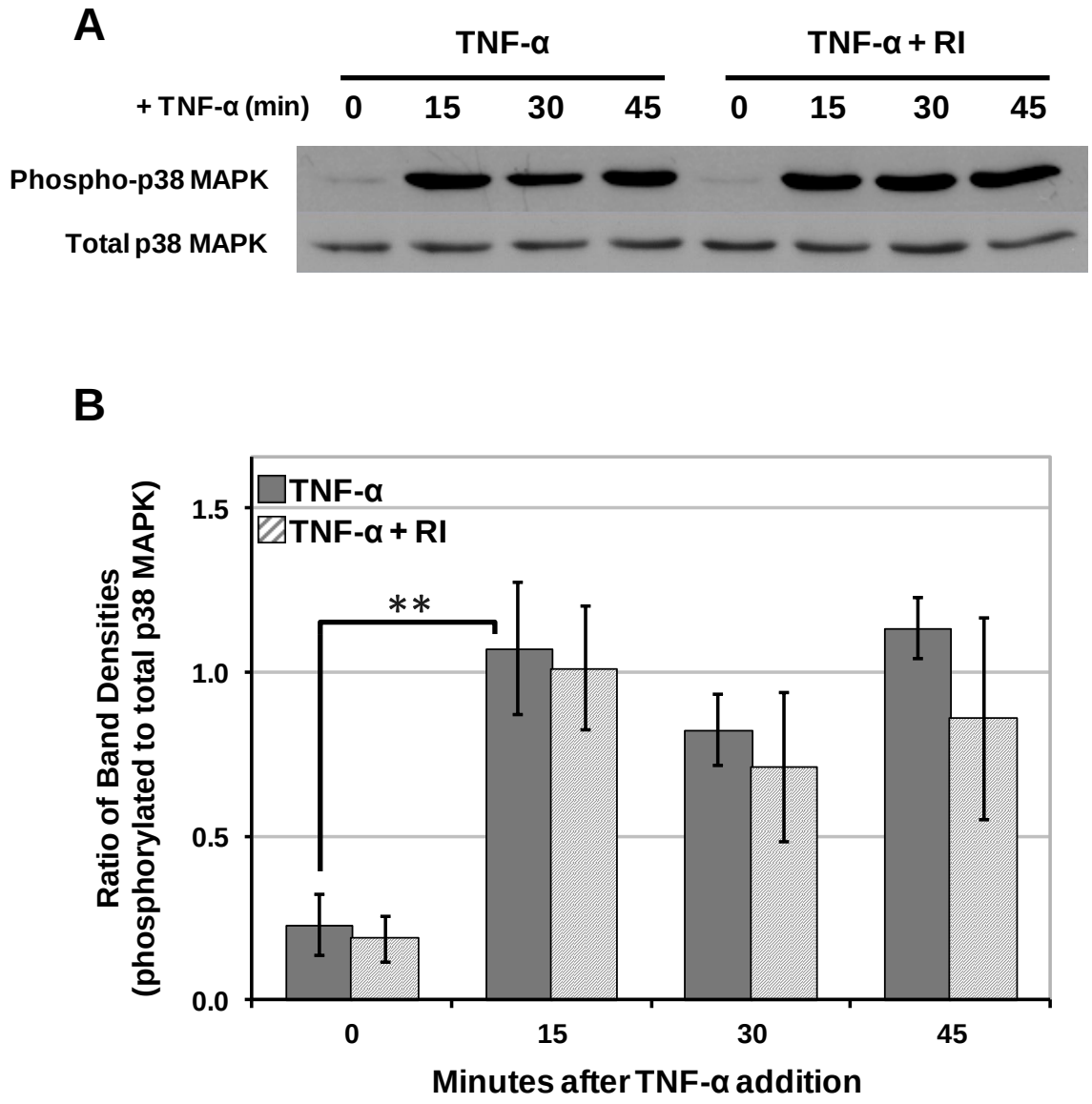

Figure 2.3: TNF-α-induced phosphorylation of p38 MAPK in Caco-2 cells is not affected by the ROCK inhibitor. Cells were seeded in FN-coated plates and pre-treated with 40 μM ROCK inhibitor prior to the addition of TNF-α (10 ng/mL) as previously described. The cells were then incubated for the indicated times and lysed. Cytoplasmic fractions were separated by SDS-PAGE and analyzed by Western blot. A representative blot is shown **(A)** with results of the band densitometry from four independent experiments **(B)**. Bars represent the means of the ratios (± 1 SEM) of phosphorylated p38 MAPK band density to total p38 MAPK band density. (** $p < 0.01$)

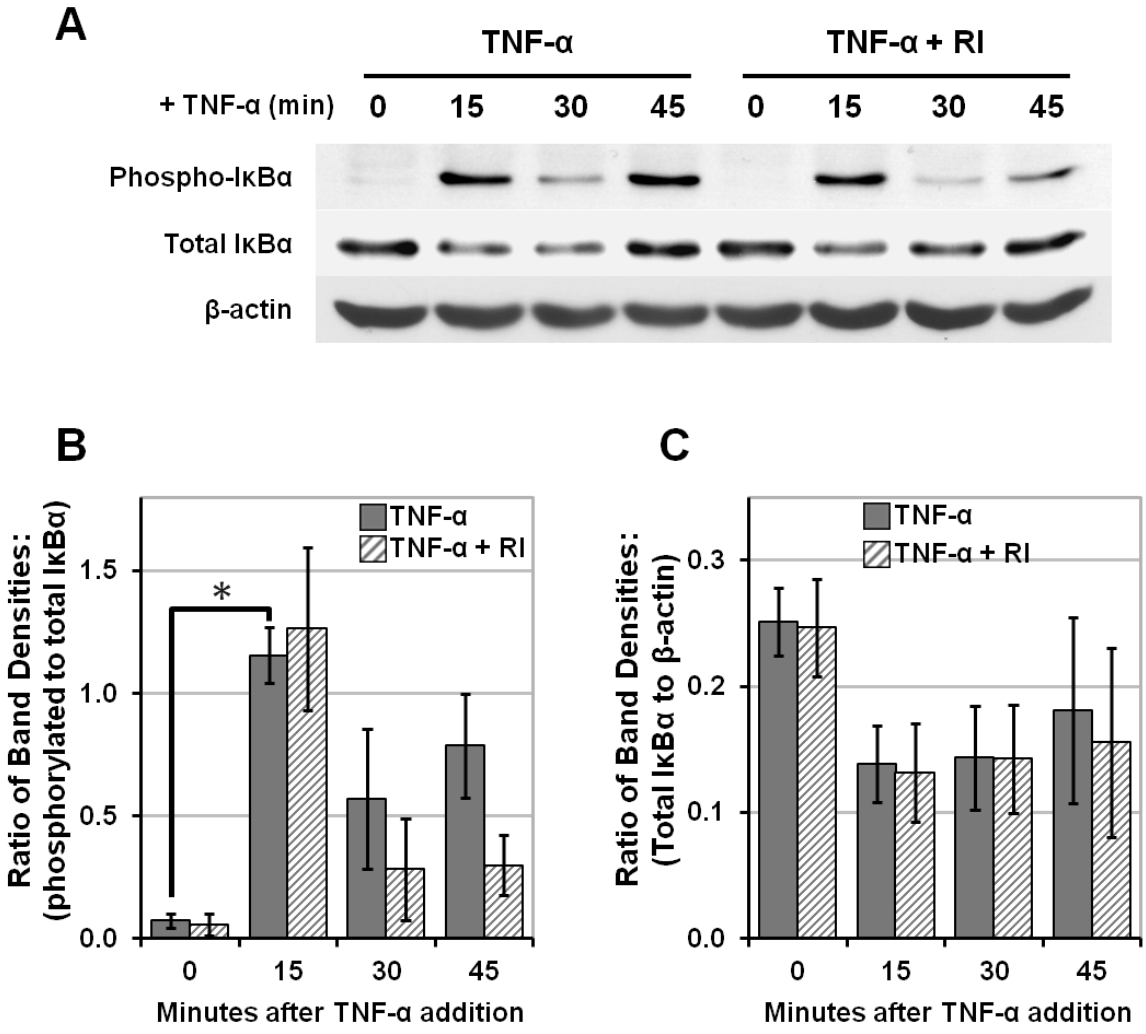

Figure 2.4: Inhibiting ROCK has little or no effect on IκBα phosphorylation and degradation in TNF-α-stimulated Caco-2 cells. Cells were cultured with 40 μM ROCK inhibitor and 10 ng/mL TNF-α and samples collected as described in Figure 2.3. The levels of phosphorylated IκBα, total IκBα, and total β-actin were examined by Western blot. A representative blot is presented here **(A)**. Graphs show the means ± 1 SEM of phosphorylated IκBα band densities normalized to total IκBα **(B)** and of total IκBα band densities normalized to total β-actin **(C)** from three separate experiments. TNF-α elicited a significant response in IκBα phosphorylation (* $p < 0.01$). However, no statistically significant differences were found between cells pre-treated with ROCK inhibitor and those not pre-treated.

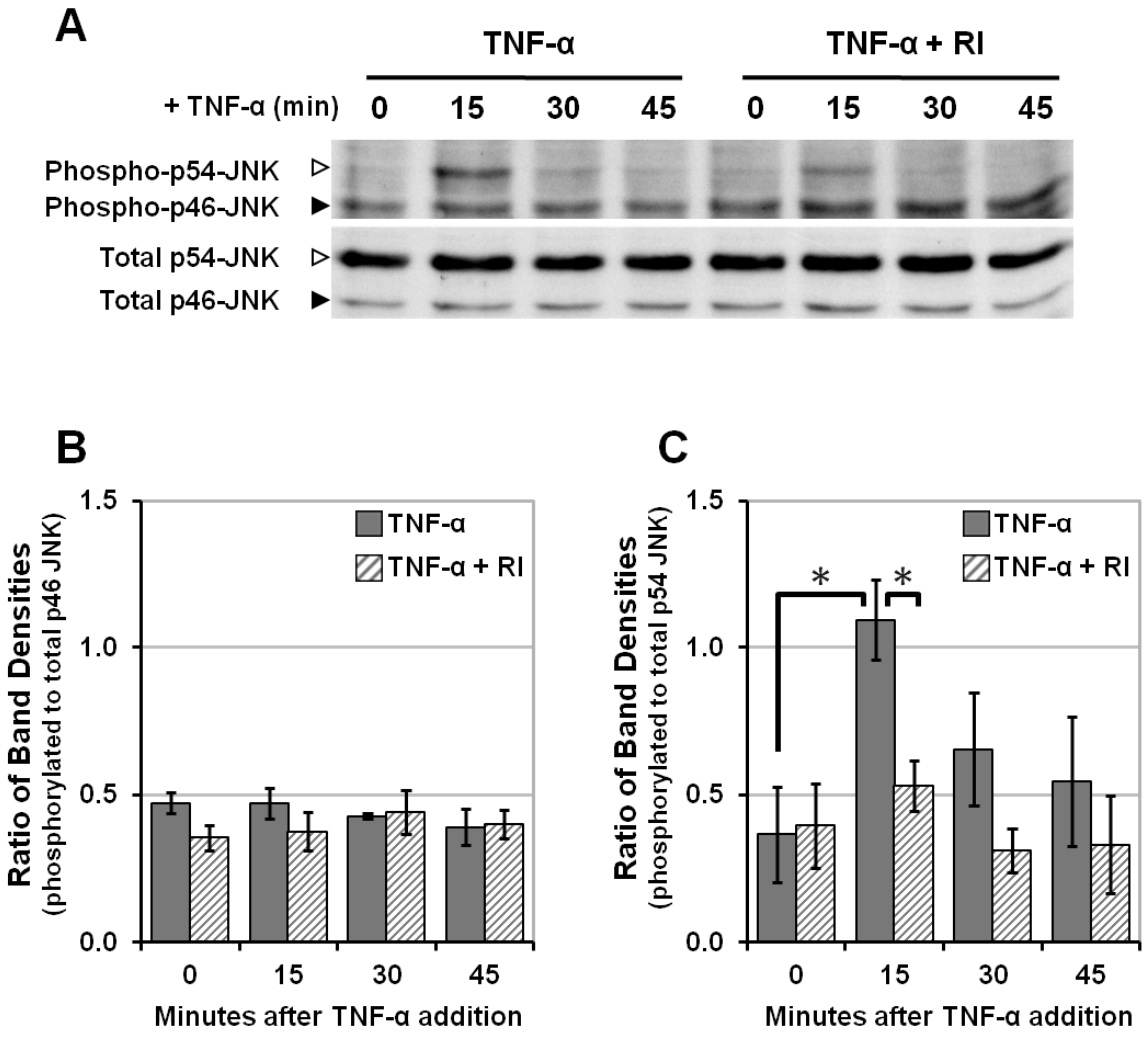

Figure 2.5: The effect of inhibiting ROCK on JNK phosphorylation in Caco-2 cells treated with TNF-α. Caco-2 cells were seeded in 12-well plates, pre-treated with 40 µM ROCK inhibitor, and incubated with 10ng/mL TNF-α for the specified times as in Figure 2.3. Cytoplasmic levels of phosphorylated JNK and total JNK were determined by Western blot. **(A)** A representative blot is shown with open arrowheads pointing to bands of the p54 JNK isoform and closed arrowheads to the p46 JNK isoform. The ratios of phosphorylated JNK band densities to total JNK were calculated for each isoform separately, and the means (± 1 SEM) of these ratios from three independent experiments are shown for both p46-JNK **(B)** and p54-JNK **(C)**. Significant differences between the indicated treatment groups are marked with asterisks (* $p < 0.05$).

4. Discussion

TNF-α plays a central role in IBD and is the target of the most current treatments for this disease [177]. Despite the advanced nature of these treatments, there are still risks of potentially serious side-effects, such as the activation of latent infections, particularly tuberculosis [177]. Expanding our knowledge of how TNF-α and other pro-inflammatory cytokines, such as IL-1β, promote inflammation in the intestinal mucosa is essential if new therapies are to be developed. As important components of the intestinal mucosa, IEC can produce several immunoregulatory signaling molecules, including CXCL8 [49-51, 114, 219], a major chemoattractant and activator of neutrophils. Elevated levels of CXCL8 are found in IBD and may play an important role in the pathogenesis of this disease [143]. Therefore, examining the regulatory mechanisms of cytokine-stimulated CXCL8 production may provide key clues in the search for new treatments.

In this study, we present evidence for the involvement of the Rho-associated coiled-coil containing kinase, ROCK, in the regulation of the CXCL8 response in IEC. Firstly, we found that inhibiting ROCK in intestinal epithelial Caco-2 cells resulted in a marked decrease in TNF-α-induced CXCL8 secretion, which corresponded to a similar decrease in CXCL8 mRNA levels. Further investigation of the role of ROCK in TNF-α signal transduction in these cells showed that the ROCK inhibitor suppressed the TNF-α-stimulated phosphorylation of the p54 isoform of JNK, while having no effect on the TNF-α-induced activation of p38 MAPK or IκBα phosphorylation and degradation. We also confirmed a prior result showing the effect of 40 μM ROCK inhibitor on IL-1β-induced CXCL8 mRNA expression using real-time RT-PCR, which was previously

determined by semi-quantitative RT-PCR and agarose gel electrophoresis [176]. Additionally, the efficacy of a reduced dose of 40 μM ROCK inhibitor in suppressing IL-1β-stimulated CXCL8 secretion was verified, an effect that was previously observed at 100 μM ROCK inhibitor [176, 220, 221].

The CXCL8 promoter contains binding sites for both NF-κB and AP-1 [107, 222], and several studies have shown that these transcription factors, alone or together, can activate CXCL8 mRNA transcription [197]. Additionally, Rho/ROCK signaling has been found to mediate the activation of both of these transcription factors under various conditions [123, 173, 175, 209, 223, 224]. Here we report that inhibiting ROCK resulted in a corresponding reduction in TNF-α-induced JNK phosphorylation in Caco-2 cells, while IκBα phosphorylation and degradation was not affected by ROCK inhibition. Because inhibiting ROCK also led to a significant reduction in TNF-α-stimulated CXCL8 mRNA expression and protein secretion, these results suggest that, under these conditions, the action of ROCK in CXCL8 production is probably mediated through AP-1 and not NF-κB. This is in accord with previous results in our laboratory that showed that inhibiting ROCK in IEC cell lines treated with IL-1β resulted in a similar suppression of JNK activation, while having no effect on IKK kinase activity, IKK phosphorylation, or IκBα phosphorylation and degradation [176, 220, 221]. Further evidence for the role of ROCK in JNK activation and CXCL8 production comes from a study by Shimada and Rajagopalan [209] in which inhibiting ROCK in LPA-treated HUVECs abrogated phosphorylation of both JNK and c-Jun in addition to suppressing LPA-induced CXCL8 mRNA expression and secretion. Furthermore, that study established that NF-κB activation in LPA-stimulated HUVECs was also ROCK-

dependent, although in this case, CXCL8 production was shown to be regulated independently of NF-κB. Segain *et al.* [123] also demonstrated that ROCK could play a role in the activation of the NF-κB pathway. In that study, ROCK inhibition was shown to block TNF-α-induced IκBα degradation and NF-κB nuclear localization in peripheral blood mononuclear cells isolated from patients with CD. Inhibiting ROCK also reduced IKKα activation and IκBα degradation in the monocytic THP-1 cell line stimulated with IL-1β. While much is still unknown, it appears that ROCK may be an important regulator of AP-1-driven responses and sometimes NF-κB-driven responses, although which pathway is affected by ROCK seems highly dependent upon the conditions and cell type.

The p38 MAPK has also been shown to regulate CXCL8 production, both at the transcriptional and post-transcriptional level [203, 204]. Here we show that TNF-α induced a 9-fold increase in p38 MAPK phosphorylation. This effect was unchanged by pre-treating the cells with ROCK inhibitor, suggesting that p38 MAPK does not play a part in the ROCK-dependent regulation of CXCL8. In addition to transcriptional control, p38 MAPK can also phosphorylate ARE-binding proteins and thus, has an effect on mRNA stability mediated by these proteins [205]. However, in TNF-α-stimulated Caco-2 cells, ROCK inhibition had no effect on CXCL8 mRNA stability (see Chapter 3), supporting the assertion that p38 MAPK is not involved in ROCK-mediated CXCL8 production in these cells.

Until the recent report from our laboratory [176], ROCK had not been identified as a possible mediator of JNK-mediated transcriptional control of CXCL8 responses in IEC. In that report, ROCK inhibition was shown to significantly suppress the activation

of both the p46 and p54 isoforms of JNK in IL-1β-treated Caco-2 cells and IEC-6 cells, indicating that ROCK may be an important player in IL-1-to-JNK signaling. Interestingly, TNF-α treatment had no effect on the phosphorylation of p46 JNK in Caco-2 cells. However, phosphorylation of the p54 isoform was increased 4.7-fold by TNF-α treatment. This isoform-specific activation was significantly reduced by pretreatment with the ROCK inhibitor, suggesting that ROCK also plays an important part in TNF-α-to-JNK signaling for optimal CXCL8 secretion by IEC.

The implications of the preferential activation of p54 JNK in these cells are, as yet, unclear. However, due to differences in substrate binding affinities among the JNK isoforms, this may provide a mechanism that allows for the activation of a specific subset of AP-1 proteins and/or other JNK-mediated responses. For instance, p54 JNK has been shown to bind c-Jun with a 25-fold greater affinity than p46 JNK [225], so the activation of the p54 isoform by TNF-α may be a way to selectively regulate transcription of genes under the control of c-Jun-containing dimers, such as c-Jun/c-Fos. JNK is also known to phosphorylate several other transcription factors, including Elk-1 and ATF2 [226], and further experimentation is required to determine the particular JNK substrate(s) that may be involved in the regulation of TNF-α-induced CXCL8 secretion in IEC. Because of the transient nature of kinase-substrate binding, a full survey of a kinase's substrates can be difficult. Here, a phosphoproteomic approach may be best in which extracts from TNF-α-stimulated cells treated with or without a JNK inhibitor would be enriched for phosphoproteins using metal oxide affinity chromatography (MOAC) [227], followed by mass spectrometry to identify substrate proteins.

Also of interest is the difference in the profiles of JNK activation by IL-1β versus TNF-α. While increased phosphorylation was limited to p54 JNK in TNF-α-treated Caco-2 cells, our previous experiments showed that exposure to IL-1β resulted in increased phosphorylation of both p46 and p54 JNK isoforms [176]. At first glance, this might seem to imply that JNK-mediated responses induced by TNF-α may be a subset of those induced by IL-1β, and this may be true. However, there are at least eight splice variants of JNK1/2, four of which are approximately 54 kDa [164]. Thus, it is possible that the observed increase in phosphorylation of p54 JNK in IEC may be a result of the selective phosphorylation of specific p54 JNK variants or sets of variants, such that TNF-α may activate an entirely different p54 variant than does IL-1β. Such non-redundant activation of JNK isoforms could explain some of the differences in IEC responses to either of these cytokines as well as suggesting a potential mechanism for IL-1β / TNF-α synergistic effects [95].

Another point to note is that ROCK inhibition did not result in complete suppression of TNF-α-induced CXCL8 secretion or mRNA expression. This is not surprising considering that TNF-α activates both NF-κB and AP-1 pathways simultaneously, which are both known to regulate CXCL8 transcription [107, 187, 228]. The suppression of JNK signaling by the ROCK inhibitor probably accounts for the observed 46% reduction in CXCL8 secretion and 82% reduction in CXCL8 mRNA, while the inability of the ROCK inhibitor treatment to affect IκBα phosphorylation and degradation in our experiments suggests that the remaining uninhibited CXCL8 production might be mediated by NF-κB. It is also possible that the residual CXCL8 levels are induced or enhanced through the action of p38 MAPK, which we also found to

be activated by TNF-α independently of ROCK. In fact, CXCL8 secretion and mRNA expression in LPA-stimulated HUVECs have been shown to be dependent on p38 MAPK but completely independent of NF-κB [209]. Furthermore, inhibiting p38 MAPK in LPA-treated HUVECs resulted in only a partial reduction in CXCL8 production, and, interestingly, complete suppression of CXCL8 required the concurrent inhibition of both p38 MAPK and JNK. A similar multiple-pathway regulatory mechanism is likely at play in Caco-2 cells as well, although it is unclear which combination of pathways is responsible for the observed TNF-α-induced CXCL8 expression. Similar to what was found in LPA-stimulated HUVECs [209], it may be the case in Caco-2 cells that CXCL8 production is under control of p38 MAPK and JNK with p38 MAPK being responsible for the observed residual CXCL8 expression when JNK activation is suppressed by the ROCK inhibitor. Alternatively, the residual CXCL8 expression may be due to NF-κB, since this pathway was not affected by the ROCK inhibitor, which suggests that a combination of NF-κB and JNK are required for the full expression of CXCL8 in TNF-α-stimulated Caco-2 cells. Furthermore, it is also possible that all three pathways, JNK, NF-κB, and p38 MAPK act together or in concert with some as yet unidentified pathway to induce CXCL8.

ROCK is an important regulator of cell movement and cytoskeletal rearrangement [172, 229, 230]. Because cell movement is essential for the wound healing process, it is likely that the edges of gastrointestinal ulcerations, which are commonly seen in IBD, may be areas of high ROCK activity. Furthermore, our results suggest that ROCK could also play a role in the cytokine responses of IEC, particularly those at the edges of ulcerations.

In our experiments, the Caco-2 cells were treated with TNF-α three hours after plating and were sub-confluent throughout the entire duration. During this period, the cells attached, spread, and began migrating toward one another, processes that require the formation and turnover of focal adhesions and stress fibers and that are also known to involve both ROCK and integrins [172]. Similarly, during healing, IEC at the wound edge also spread and migrate towards one another in a Rho/ROCK dependent manner [101, 231-233]. When considered along with these facts, the results of our experiments could suggest that ROCK may have the additional role of enhancing the TNF-α- and IL-1β-stimulated CXCL8 responses in wound-edge IEC. In this scenario, the subsequent increase in CXCL8 near the wound edge would direct neutrophils to the wound opening where they could combat any invading microbes. The possibility of such a multifaceted role suggests that ROCK may be an important and essential player in the overall response of IEC during intestinal wounding and inflammation.

Despite the incomplete suppression of CXCL8 secretion observed in Caco-2 cells, ROCK inhibition may still be an attractive treatment option for IBD. Reduced CXCL8 production by IEC, particular at the wound edge, would likely mean fewer neutrophils infiltrating inflamed and ulcerated intestinal tissues. The resultant decrease in pro-inflammatory cytokines, as well as tissue-damaging substances, that are produced by neutrophils could shift the balance of immune regulatory signals toward anti-inflammatory and healing responses. While ROCK inhibition in IEC could be effective treatment in and of itself, a study by Segain et al. [123] has shown that the ROCK inhibitor can also significantly suppress the secretion of TNF-α by lamina propria mononuclear cells isolated from patients with CD and rats with TNBS-induced colitis.

This finding corresponded with a significantly reduction in inflammation in the intestinal tissues of rats with TNBS colitis when the ROCK inhibitor was administered. In that study, however, the role of IEC was not considered, and the observed reduction in inflammation may have resulted, at least in part, from the blockade of ROCK-mediated responses in IEC. Taken together, these results indicate that the ROCK inhibitor could be a potent anti-inflammatory agent that reduces pro-inflammatory activity in multiple cell types and that ROCK inhibition could be a highly effective treatment strategy for IBD.

Chapter 3

The Role of ROCK in the Differential Regulation of Cytokine-Induced Production of CXCL8 Compared to CCL2 and CCL20 in Intestinal Epithelial Cells

1. Introduction

Chemokines are a group of cytokines, consisting of small, soluble, secreted proteins that act as chemoattractants and activators of leukocytes [100]. Chemokines are organized into four subfamilies, C, CC, CXC, and CX_3C, based on the arrangement of conserved cysteine residues with almost all chemokines falling into either of the two major subfamilies: CXC or CC [98, 234]. Neutrophils respond mainly to CXC chemokines, in particular CXCL8, although they are also acted upon by CXCL1 and CXCL2 [100, 101, 108]. In contrast, monocytes and macrophages are mainly, but not exclusively, responsive to CC family chemokines such as CCL2 [100, 101]. The modes of action upon other leukocytes are not as well delineated between the CXC and CC subfamilies. For instance, CXCL10 exerts its effects on T_H1 lymphocytes and natural killer cells [98, 100], whereas CCL20 is known to act upon mucosal-homing T lymphocytes and immature dendritic cells [101, 113].

Inflammatory Bowel Disease (IBD) is characterized by the aberrant expression of many immunomodulatory molecules, including IL-1β, TNF-α, IL-6 [235], as well as

many chemokines, such as CXCL1, CXCL8, CCL2, and CCL20 [141]. CXCL8 is found at especially high levels in IBD compared to many other chemokines [74, 141], and accordingly, high numbers of neutrophils are also found in IBD-affected tissues [143]. IEC form a major component of the mucosal immune system, and thus, IEC isolated from IBD patients have been found to produce many of these aberrantly expressed chemokines at elevated levels [46, 74, 124, 144, 184]. Furthermore, IEC cell lines stimulated with either IL-1β or TNF-α have also been shown to produce many of these same chemokines [46, 50, 51]. Because IEC have the ability to produce chemokines and because the IEC microenvironment in IBD tissues contains the known inducers of chemokine production, IL-1β and TNF-α [78, 117, 119-121, 124, 125], it is likely that IEC make a considerable contribution to the increased levels of chemokines found in IBD.

The TNF-α signal is transduced through a highly complex network of intracellular signaling pathways. One of the main modes of action of TNF-α signaling is the initiation of transcription of immediate early genes through the activation of the NF-κB and AP-1 transcription factors [87, 187]. One such immediate early gene is the chemokine CXCL8, which can be activated by NF-κB alone or in combination with AP-1 in a synergistic manner [106, 107, 197]. The CC chemokine, CCL2, is another gene whose transcription and secretion can be induced by TNF-α [112]. Similar to CXCL8, CCL2 transcription is also regulated by NF-κB and AP-1 as well as by other transcription factors [112, 197]. Aside from NF-κB and AP-1, TNF-α is known to activate several other signaling pathways. These include the p38 MAPK pathway, which is known to affect chemokine production by regulating AU-rich element (ARE)-binding proteins and subsequently increasing mRNA stability [87, 187, 205]. In addition, TNF-α has also been found to

activate the PI3K/Akt pathway, the ERK pathway, and the protein kinase C (PKC) pathway [86, 154, 187, 236].

In addition, signaling through ROCK has also been found to be activated by TNF-α stimulation, and recently, evidence for a role for ROCK in TNF-α-associated inflammation has been growing. Segain *et al*. [123] found that inhibiting ROCK reduced the levels of inflammation in the colons of rats with TNBS-induced colitis. Furthermore, they found that ROCK inhibition blocked the TNF-α-induced degradation of IκBα in peripheral blood mononuclear cells and also blocked TNF-α-induced nuclear localization of NF-κB as shown by immunofluorescence. In experiments in our laboratory, inhibiting ROCK significantly reduced TNF-α-stimulated mRNA expression and secretion of CXCL8 in IEC cell lines and also suppressed the TNF-α-induced activation of MKK4 and p54 JNK [237, Chapter 2]. Classically, ROCK has been implicated in the regulation of processes involving integrins and the cytoskeleton, such as cell adhesion, stress fiber formation, actin-myosin contractility, and integrin signaling [170, 172, 238, 239]. Because ROCK is known to play roles in both integrin and TNF-α signaling, it may be possible that ROCK could act as a convergence point for crosstalk between of integrin and TNF-α signals.

In addition to showing that ROCK is involved in the TNF-α-induced production of CXCL8 in Caco-2 and DLD-1 cells, studies from our lab have also indicated that ROCK is involved in the IL-1β-stimulated production of CXCL8 in Caco-2 cells and in the IL-1β-stimulated production of CCL2 in rat-derived IEC-6 cells [176, 237, Chapter 2]. These results suggest that ROCK may play a role in the regulation of chemokine responses by IEC, and thus may be a potential therapeutic target for the

treatment of IBD. Therefore, to further elucidate the role of ROCK in IEC chemokine responses, we extended our investigations by examining the function of ROCK in cytokine-induced CCL2 production in the human-derived Caco-2 cell line and also in the cytokine-induced production of CCL20, another IBD-associated CC chemokine.

2. Materials and Methods

2.1 Inhibitors.

Wortmannin, a specific inhibitor of PI3K and triciribine, a specific inhibitor of Akt/protein kinase B were purchased from Sigma-Aldrich (St. Louis, MO). GF109203X, a specific inhibitor of conventional protein PKC isoforms (α, β, γ, δ, and ε subtypes [240, 241]) was obtained from Enzo Life Sciences (Farmingdale, NY) as was the transcription inhibitor, actinomycin D, and the ROCK inhibitor, Y-27632. PD98059, a specific inhibitor of MEK1/2, was obtained from EMD Biosciences (Billerica, MA).

2.2 Cell culture conditions.

The human colonic epithelial Caco-2 cell line (ATCC) was cultured in FBS-DMEM and maintained at 37°C in a humidified 10% CO_2 atmosphere as described in Chapter 2. For experiments, Caco-2 cells were seeded in FN-coated 6-, 12-, and 24-well plates as described in Chapter 2. The cells were allowed to attach for 1 h at 37°C, and then the medium was aspirated and replaced with serum-free ITS-DMEM or ITS-DMEM containing 40 μM Y-27632 ROCK inhibitor. After an additional 2 h of incubation, the cells were treated with 10 ng/mL rhTNF-α (R&D Systems, Minneapolis, MN) and incubated for the appropriate time periods required by each experimental protocol. In some experiments, additional chemical inhibitors were added prior to treatment with TNF-α as indicated in the following sections.

2.3 Determination of chemokine levels in culture supernatants.

Caco-2 cells were seeded in FN-coated 24-well plates (2×10^5 cells/well) and treated with Y-27632 as described above. After pretreatment with the ROCK inhibitor for 2 h, either 10 ng/mL TNF-α or 1 ng/mL IL-1β was added to the wells, and the plates were incubated for 24 h. Culture supernatants were analyzed for chemokine content using CXCL8, CCL2, or CCL20 DuoSet ELISA kits (R&D Systems), and the number of cells in each well was determined as described in Chapter 2. Chemokine levels were normalized to cell counts and expressed in pg/10^5 cells.

In experiments investigating which signaling pathways might be involved in ROCK-mediated regulation of chemokine secretion, the cells were also pretreated with the inhibitors listed in Table 3-1, either separately or in combination with the ROCK inhibitor. For Wortmannin, the inhibitor or vehicle alone was added to each well after the cells had been incubated for 1 h in the presence of the ROCK inhibitor. This was followed by an additional 1 h incubation period before treatment with TNF-α, such that the total time of pretreatment with ROCK inhibitor was 2 h, maintaining consistency with previous experiments. For the remaining inhibitors, the cells were pretreated with ROCK inhibitor for 1.5 hr before the addition of the second inhibitor. The cells were then incubated for an additional 0.5 h, again for a total of 2 h of treatment with the ROCK inhibitor prior to treatment with TNF-α. After the addition of TNF-α, the plates were incubated for 24 h, and levels of secreted chemokines were determined as described above.

Table 3-1. Inhibitor treatments

Inhibitor	Time of treatment prior to TNF-α addition	Final [inhibitor]	Vehicle (% DMSO in ITS-DMEM)	Final % DMSO in culture
Wortmannin	1 h	10, 100 nM	0.4	0.005
Triciribine	30 min	1, 10 μM	2	0.05
PD98059	30 min	25 μM	5.4	0.13
GF109203X	30 min	2.5 μM	5.4	0.13

2.4 Quantification of relative mRNA levels.

The relative levels of CXCL8 and CCL2 mRNAs in total RNA isolated from TNF-α-stimulated Caco-2 cells were determined using real-time RT-PCR as described in Chapter 2. To do so, the cells were first seeded in FN-coated 6-well culture plates at a density of 1×10^6 cells/well. The cells were then pretreated with ROCK inhibitor or medium alone for 2 h before treatment with either 10 ng/mL TNF-α or 1 ng/mL IL-1β for 6 h. Total RNA was isolated from the cells and converted to cDNA, which was then analyzed by real-time PCR using CXCL8, CCL2, and GAPDH primer sets (SABiosciences, Valencia, CA).

2.5 Examination of mRNA decay rates.

The decay rates of CXCL8 and CCL2 mRNAs were determined by measuring relative transcript levels over a time course of 2 h. Caco-2 cells were pretreated with or without the ROCK inhibitor and treated with TNF-α as described in the previous section. After incubation for 6 h with TNF-α, the transcriptional inhibitor, actinomycin D, was added to

all wells at a concentration of 10 μg/mL. The plates were returned to the incubator, and total RNA was extracted from the cells after 0, 0.5, 1, and 2 h. Relative mRNA levels were determined using real-time RT-PCR as previously described.

2.6 Immunoblot analysis of ERK phosphorylation.

Cytoplasmic extracts were prepared from Caco-2 cells treated with the ROCK inhibitor and TNF-α as described in Chapter 2. The extracts were analyzed for phospho-ERK and total ERK using Western blot (Chapter 2), and the levels of phospho-ERK were determined by band densitometry and normalized to total ERK band densities. Antibodies against phospho-ERK (p44/p42) and total ERK (p44/p42) were purchased from Cell Signaling Technologies (Beverly, MA), as were the HRP-conjugated anti-mouse and anti-rabbit antibodies used for detection.

2.7 Statistics

Statistical significance was determined using ANOVA followed by Fisher's protected least significant difference post-hoc test with the level of significance set at $p < 0.05$. A minimum of three replicates ($n \geq 3$) were run for each experiment.

3. Results

3.1 The effect of inhibiting ROCK on chemokine secretion by IL-1β- and TNF-α-stimulated Caco-2 cells

IEC taken from IBD patients and IEC cell lines treated with inflammatory stimuli have both been shown to produce the chemokine CXCL8, a major neutrophil chemoattractant and activator with increased expression in IBD [49, 74, 143]. In previous reports, we have shown that ROCK is required for optimal secretion of CXCL8 in the Caco-2 human colonic epithelial cell line when stimulated with either IL-1β or TNF-α [176, 237, Chapter 2], two important pro-inflammatory cytokines also found at elevated levels in IBD [78, 132]. To further elucidate the role that ROCK might play in IEC inflammatory responses, we began by expanding our investigations to include CCL2 and CCL20, two other important chemokines with elevated expression in IBD [74, 145, 146].

In order to determine if ROCK is involved in the regulation of CCL2 or CCL20 secretion in IEC, Caco-2 cells were added to the wells of FN-coated culture plates and pretreated with the ROCK inhibitor, Y-27632, at a concentration of 40 μM as before (Chapter 2). The cells were then stimulated with TNF-α (10 ng/mL) or IL-1β (1 ng/mL) for 24 hours, and the resulting culture supernatants were tested by ELISA. We first analyzed the supernatants for accumulated CXCL8 to confirm the reproducibility of our prior results and to establish a baseline response for the sake of making more accurate comparisons within this experiment set. As expected, CXCL8 secretion in Caco-2 cells was greatly increased by the addition of either TNF-α (Figure 3.1A) or IL-1β (Figure 3.2A) compared to untreated controls, while the cells pre-treated with the ROCK

inhibitor yielded significantly reduced cytokine-stimulated CXCL8 secretion as compared to cells treated with TNF-α alone.

Because CCL2 is also produced by IEC in response to pro-inflammatory signals [51, 146], we hypothesized that inhibiting ROCK in Caco-2 cells would also result in a suppression of CCL2 secretion similar to that of CXCL8. Furthermore, this hypothesis followed from previous results in our laboratory that showed that inhibiting ROCK suppressed IL-1β-stimulated CCL2 mRNA expression and secretion in rat-derived IEC-6 cells [176]. To the contrary of this hypothesis, Caco-2 cells pretreated with the ROCK inhibitor yielded a significant 2-fold enhancement of TNF-α-induced CCL2 secretion (Figure 3.1B) and a significant 1.5-fold enhancement of IL-1β-induced CCL2 secretion (Figure 3.2B) as compared to levels in cells treated with TNF-α or IL-1β alone. This result led us to ask if this effect was specific to CCL2 or whether it might extend to other C-C chemokines. To answer this, the culture supernatants were next analyzed for CCL20, a chemokine that not only is expressed at abnormally high levels in IBD [114, 115] but also has been shown to be produced almost exclusively by IEC in inflamed intestinal mucosae [114]. Similar to CCL2 secretion, there was a 2-fold increase in TNF-α-induced CCL20 secretion (Figure 3.1C) and a 1.4-fold increase in IL-1β-induced CCL20 secretion (Figure 3.2C) by Caco-2 cells treated with the ROCK inhibitor over cells without the inhibitor. This suggests that the activation of ROCK somehow leads to a suppression of IL-1β- and TNF-α-induced C-C chemokine responses by IEC.

3.2 ROCK inhibition also results in the differential regulation of CCL2 and CXCL8 at the mRNA level in Caco-2 cells treated with TNF-α or IL-1β

We have previously shown that ROCK-dependent CXCL8 production is regulated at the mRNA level in Caco-2 cells treated with either IL-1β or TNF-α [176, Chapter 2]. Thus, we next set out to determine whether the opposing regulatory mechanism that partially suppresses CCL2 and CCL20 secretion also affected mRNA levels using CCL2 as a representative from this group. To do this, Caco-2 cells were seeded in FN-coated culture plates, treated with the ROCK inhibitor and TNF-α as specified above, and analyzed by real-time RT-PCR.

Similar to our experiments examining chemokine secretion, the samples were first analyzed for CXCL8 mRNA for comparison within this set of experiments. Consistent with the results reported in Chapter 2, TNF-α treatment elicited a 30-fold up-regulation of CXCL8 mRNA levels, which was significantly reduced with ROCK inhibition (Figure 3.3A). A subsequent analysis of these samples showed that TNF-α induced a significant 8-fold increase in CCL2 mRNA levels as compared to untreated controls, which was boosted to nearly 14-fold over untreated controls when TNF-α and the ROCK inhibitor were administered together (Figure 3.3B). The expression of CCL2 mRNA in IL-1β-stimulated Caco-2 cells was likewise enhanced to approximately 15-fold over untreated controls by the ROCK inhibitor versus an 8-fold increase in cells treated with IL-1β alone (Figure 3.4). In both cases, whether TNF-α or IL-1β treatment, concomitant ROCK inhibition resulted in the enhancement of CCL2 mRNA levels.

The finding that the ROCK inhibitor enhances CCL2 and CCL20 responses directly contradicted our initial hypothesis that predicted a suppression similar to that

seen for CXCL8. Because of the surprising nature of this finding, our next objective was to confirm that the observed enhancement was not due to an off-target side-effect of the ROCK inhibitor. As discussed in Chapter 2, off-target effects can occur at higher concentrations of the ROCK inhibitor, while at lower concentrations, the inhibitor is highly specific for ROCK [211, 212]. The 40 μM dose used in these experiments was chosen to maximize ROCK inhibition. However, somewhat lower doses can still produce significant, although not maximal, inhibition of ROCK. If the observed partial suppression of CCL2 and CCL20 responses was in fact mediated by ROCK, then treating cells with a reduced dose of 10 μM ROCK inhibitor would still result in a significant enhancement, whereas if the effect was mediated by an off-target kinase with a higher half-maximal inhibitory concentration (IC_{50}), such as PKC, protein kinase A, or the citron kinase [211], a dose of 10 μM should produce little or no enhancement.

To test this, CCL2 mRNA expression was examined in TNF-α-stimulated Caco-2 cells pretreated with concentrations of ROCK inhibitor ranging from 0 to 100 μM (Figure 3.5). In accord with the hypothesis that the observed effect was indeed due to ROCK-specific inhibition, the lowest dose (10 μM) of ROCK inhibitor produced a significant enhancement of TNF-α-stimulated CCL2 mRNA expression. In fact, there was no significant difference between the enhancement produced with 10 μM ROCK inhibitor and that produced with the 40 μM concentration, which is strong evidence that the enhancement effect was due to the specific inhibition of ROCK. Interestingly, increasing the dose of the ROCK inhibitor to 100 μM abolished the enhancement effect. This return of CCL2 mRNA expression to TNF-α-only levels suggests that the activity of off-target kinases may be involved in CCL2 expression. However, since the

enhancement effect remained undiminished up to the 40 μM dose of ROCK inhibitor, it is likely that the activity of such kinases is largely or completely unaffected by the ROCK inhibitor under the conditions used in this study.

3.3 The effect of inhibiting ROCK in TNF-α-treated Caco-2 cells on CXCL8 and CCL2 mRNA stability

Having established that inhibiting ROCK results in the enhancement of TNF-α-induced CCL2 responses in Caco-2 cells, we next set out to investigate how ROCK might be effecting such disparate regulatory outcomes between CXCL8 and CCL2. Our data showed that ROCK-dependent CXCL8 secretion and the ROCK-dependent attenuation of CCL2 secretion were both associated with corresponding changes in steady-state mRNA levels. Post-transcriptional control of steady-state mRNA levels through the p38 MAPK mediated modulation of deadenylation-dependent mRNA decay has been identified as a fundamental mechanism in the regulation of many cytokines, including several chemokines [242]. Therefore, our first step in dissecting the differential regulation of CXCL8 and CCL2 was to examine the effect of the ROCK inhibitor on mRNA degradation. To do this, Caco-2 cells were incubated with the ROCK inhibitor and TNF-α and then treated with actinomycin D to halt transcription. Measurements of mRNA levels over the course of a subsequent 120 minute time period showed that inhibiting ROCK had little or no effect on the decay rate of CCL2 mRNA or of CXCL8 mRNA (Figure 3.6). To note, the results do show an apparent, but slight, destabilization of CCL2 mRNA at 120 minutes in cells treated with TNF-α and the ROCK inhibitor, suggesting that inhibiting ROCK may lead to lower steady-state mRNA levels. This,

however, is completely inconsistent with our previous observation that ROCK inhibition resulted in higher steady-state CCL2 mRNA levels and is likely an anomalous reading, especially considering the small sample size (n = 2).

3.4 Investigation of the PI3K/Akt pathway for involvement in the ROCK-dependent attenuation of TNF-α-induced CCL2 secretion

Our previous results indicating that ROCK mediates JNK activation in Caco-2 cells stimulated with IL-1β or TNF-α [176, Chapter 2] support a straight-forward model of regulation in which optimal CXCL8 transcription is contingent upon the ROCK-dependent activation of JNK. At the same time, it appears that ROCK also governs a negative regulatory mechanism that has a suppressive effect on CCL2 and CCL20 expression. In light of the divergent nature of these responses, we reasoned that the suppression of CCL2 mRNA production is most likely independent of JNK, and, therefore, set out to identify additional signaling pathways involved in ROCK-mediated CCL2 expression. Both a study using HUVECs and a study using human retinal pigment epithelial cells showed that activation of the PI3K/Akt pathway is essential for CCL2 secretion in cells treated with TNF-α [153, 154]. The study with human retinal pigment epithelial cells also found that, unlike CCL2, the induction of CXCL8 secretion by TNF-α occurred independently of PI3K/Akt activation [153]. Although this pattern of regulation does not directly correspond to the ROCK-mediated regulation of CXCL8 and CCL2 seen in Caco-2 cells, the fact that the effects of PI3K/Akt activation were limited to CCL2 secretion in human retinal pigment epithelial cells prompted us to ask whether

the PI3K/Akt pathway might be playing a similar role in the ROCK-dependent regulatory mechanisms governing CCL2 secretion in Caco-2 cells.

We approached this query by first examining the effect of the specific PI3K inhibitor, Wortmannin, on the enhancement of TNF-α-stimulated CCL2 secretion by the ROCK inhibitor. As in previous experiments, Caco-2 cells were again pre-treated with the ROCK inhibitor and then incubated in the presence of TNF-α. However, in this set of experiments, the cells were also pre-treated with Wortmannin at 10 and 100 nM (or with vehicle). In the vehicle-treated group, TNF-α once again elicited a significant increase in CCL2 secretion, which was enhanced further by ROCK inhibition (Figure 3.7). This pattern of response to TNF-α and the ROCK inhibitor was unchanged when the cells were pre-treated with Wortmannin at either concentration.

While this is fairly strong evidence that PI3K/Akt signaling is not involved in regulating CCL2 secretion in TNF-α-treated Caco-2 cells, there have been reports that PI3K inhibition is not, in some cases, sufficient to abrogate the downstream activity of Akt. Studies have identified other regulatory proteins, such as p38 MAPK and PTEN, as possible PI3K-independent mediators of downstream Akt activity [243-248]. Therefore, to confirm the results seen with Wortmannin, we next tested whether directly inhibiting Akt using triciribine (Akt inhibitor V) would have an effect on CCL2 secretion in these cells. Unlike inhibiting PI3K with Wortmannin, Akt inhibition resulted in an overall reduction in TNF-α-induced CCL2 secretion, but only at the highest concentration of triciribine, 10 μM (Figure 3.8). Despite this reduction, inhibiting Akt in ROCK inhibitor-treated cells had no effect on the ROCK-dependent enhancement of TNF-α-induced CCL2 secretion, resulting in an increase proportionally equivalent to that seen in cells

treated without the Akt inhibitor (1.6-fold vs. 1.9-fold enhancement with or without the Akt inhibitor respectively; $p > 0.5$).

3.5 The effects of PKC, MEK, and ROCK inhibition on CCL2 secretion in TNF-α-treated Caco-2 cells

Several studies have shown that PKC regulates the secretion of CCL2 and/or CXCL8 under certain conditions [249-251]. In other cases, the secretion of either or both of these chemokines is under the control of ERK or PKC and ERK together [252-257]. Among these studies are reports that point toward possible roles for PKC and ERK in negative regulatory mechanisms governing cytokine secretion [251-253]. To determine whether PKC or ERK might be involved in the regulation of chemokines in IEC, each pathway was blocked separately with chemical inhibitors, and then the effects of these inhibitors on the ROCK-mediated attenuation of TNF-α-stimulated CCL2 secretion were examined. To block PKC signaling, Caco-2 cells were treated with GF109203X, a potent inhibitor of the PKCα, PKCβ, PKCγ, PKCδ, and PKCε isoforms [240, 241], while the ERK pathway was blocked using PD98059, which inhibits MEK1/2, the upstream kinases that phosphorylate and activate ERK1/2 [168, 258].

In Caco-2 cells treated with the PKC inhibitor, there was a small, but not significant, increase in TNF-α-elicited CCL2 secretion, which occurred independently of ROCK inhibition (Figure 3.9A). However, this increase should be noted as a potential sign that PKC may have a minor and/or generic role in IEC chemokine secretion. More importantly, the blockade of PKC had little or no effect on the ability of ROCK to suppress CCL2 secretion in TNF-α-treated Caco-2 cells, since the inhibition of ROCK

still resulted in a significant enhancement of TNF-α-induced CCL2 secretion when PKC was concurrently inhibited, indicating that PKC plays little or no role in CCL2 regulation.

In contrast, when MEK/ERK was inhibited, there was a significant 1.8-fold increase in TNF-α-induced CCL2 secretion over levels from cells treated with TNF-α but without the MEK/ERK inhibitor (Figure 3.9B). In addition, this increase in TNF-α-stimulated CCL2 secretion was to a level equal to that of TNF-α-stimulated CCL2 secretion from cells treated with the ROCK inhibitor. Furthermore, the MEK/ERK inhibitor had no effect on CCL2 secretion in cells treated with TNF-α and the ROCK inhibitor. Taken together, these results suggest that the MEK/ERK pathway may play an import role in the ROCK-associated control of CCL2 responses in Caco-2 cells.

3.6 Blocking PKC or MEK activity had no effect on the suppression of TNF-α-stimulated CXCL8 secretion in Caco-2 cells

While these experiments provided insight into the mechanism responsible for the partial suppression of CCL2 secretion, the question of whether MEK or PKC played any role in mediating ROCK-potentiated CXCL8 secretion had yet to be answered. Analysis of culture supernatants from the above experiments revealed that ROCK inhibition reduced TNF-α-stimulated CXCL8 secretion to the same level regardless of whether the ROCK inhibitor was administered by itself or concomitantly with the PKC inhibitor or with the MEK inhibitor (Figure 3.10). It should be noted that, in both cases, treatment with the MEK or PKC inhibitor resulted in a slight, but statistically significant, decrease in TNF-α-induced CXCL8 secretion in the absence of ROCK inhibition. However, these

decreases were not nearly of the magnitude that would be expected if either of these kinases played any substantial role in the ROCK-dependent secretion of CXCL8.

3.7 The effect of ROCK Inhibition on TNF-α-stimulated ERK phosphorylation in Caco-2 cells

Having identified the MEK/ERK pathway as a regulatory component moderating TNF-α-induced CCL2 secretion, we next sought to establish the relationship, if any, between ROCK and ERK signaling. For this, Caco-2 cells were treated with the ROCK inhibitor and TNF-α in the previously described manner, and the cells were lysed at fifteen minute intervals for 45 minutes. Western blot was then used to assess the effect of ROCK inhibition on ERK phosphorylation. In cells incubated without the ROCK inhibitor, TNF-α treatment resulted in a 2.3-fold increase in the levels of phosphorylated ERK after 15 minutes, which subsided to baseline levels by 30 minutes (Figure 3.11). In contrast, when ROCK was inhibited, ERK phosphorylation at 15 minutes was suppressed to baseline levels. This suggests that ROCK is an important component of the TNF-α signaling cascade leading to ERK activation in Caco-2 cells. However, the data also showed that ERK phosphorylation was similarly suppressed in the ROCK inhibitor treatment group in the absence of TNF-α at time zero ($p < 0.05$). Furthermore, TNF-α stimulation of the ROCK inhibitor-treated cells still resulted in a significant increase in the phosphorylation of ERK at 15 minutes compared to the zero-minute levels ($p < 0.05$). Thus, ROCK inhibition may not have affected the ability of TNF-α to stimulate ERK phosphorylation but did limit the scale of the effect such that peak levels of

phosphorylated ERK in ROCK inhibitor-treated cells only reached baseline levels of cells treated without inhibitor.

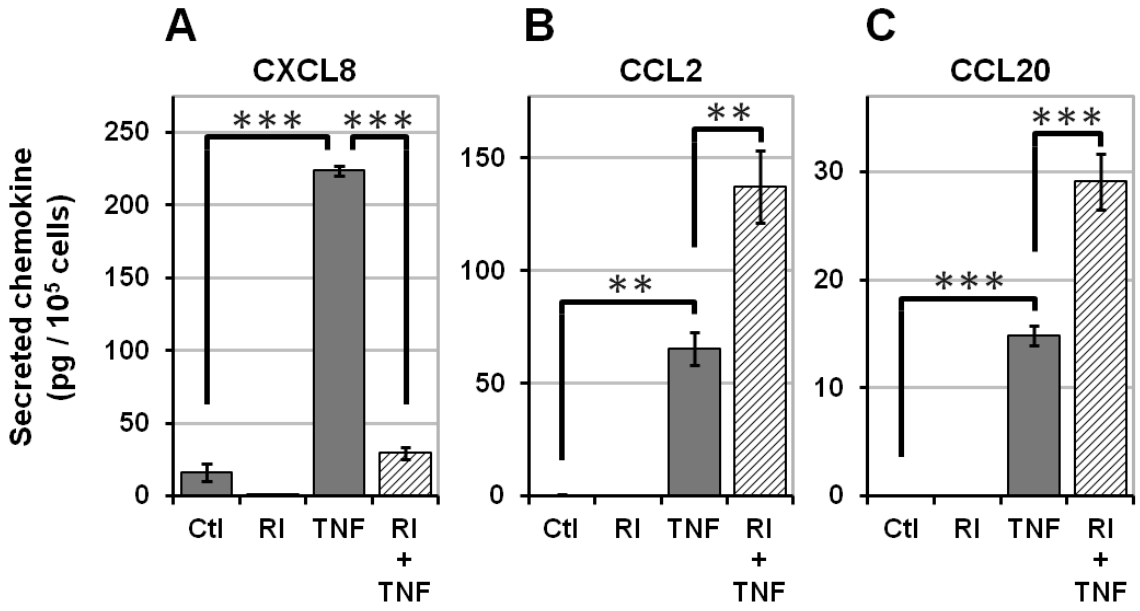

Figure 3.1: Inhibiting ROCK in TNF-α-treated Caco-2 cells results in a suppression of CXCL8 secretion but an enhancement of CCL2 and CCL20 secretion. FN-coated 24-well culture plates were seeded with Caco-2 cells in FBS-DMEM and incubated at 37°C for 1 h to allow the cells to attach. The medium was then aspirated and replaced with ITS-DMEM with or without 40 µM ROCK inhibitor (RI). The cells were returned to the incubator for 2 h and then treated with 10 ng/mL TNF-α. After an additional 24 h period of incubation, culture supernatants were collected and analyzed by ELISA for the indicated chemokines, and the number of cells in each well was determined. The resulting chemokine concentrations were then normalized to cells/well. Shown here are the averages ± 1 SEM of three replicates. (Ctl, untreated controls; ** $p < 0.01$, *** $p < 0.0001$)

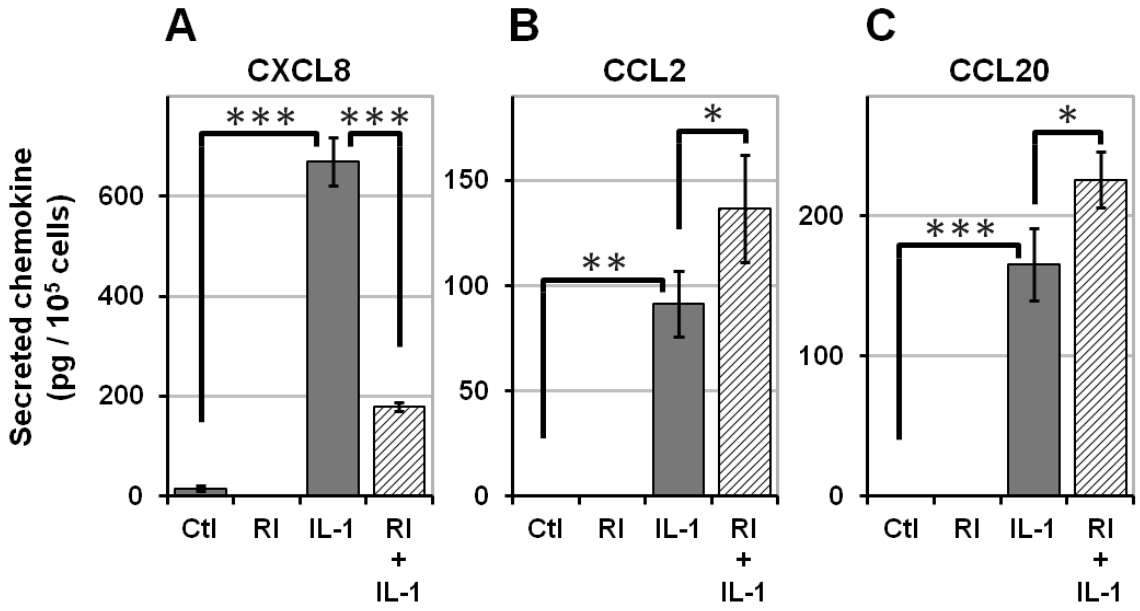

Figure 3.2: The suppression of CXCL8 secretion and the enhancement of CCL2 and CCL20 secretion are also seen when ROCK is inhibited in IL-1β-treated Caco-2 cells. Cells were plated and pre-treated with ROCK inhibitor as described in Figure 3.1. The cells were then incubated with or without 1 ng/mL IL-1β for 24 h. The concentrations of the indicated chemokines in the resultant culture supernatants were then determined by ELISA and normalized to the number of cells in each well. Bars represent the means ± 1 SEM of three independent experiments. Note: IL-1β treatments were run in the same experiments as the TNF-α treatments described in Figure 3.1. Thus, both treatments are compared to common controls, and the same control data (Ctl and RI) are displayed in both Figures 3.1 and 3.2. (Ctl, untreated controls; * $p < 0.05$, ** $p < 0.001$, ** $p < 0.0001$)

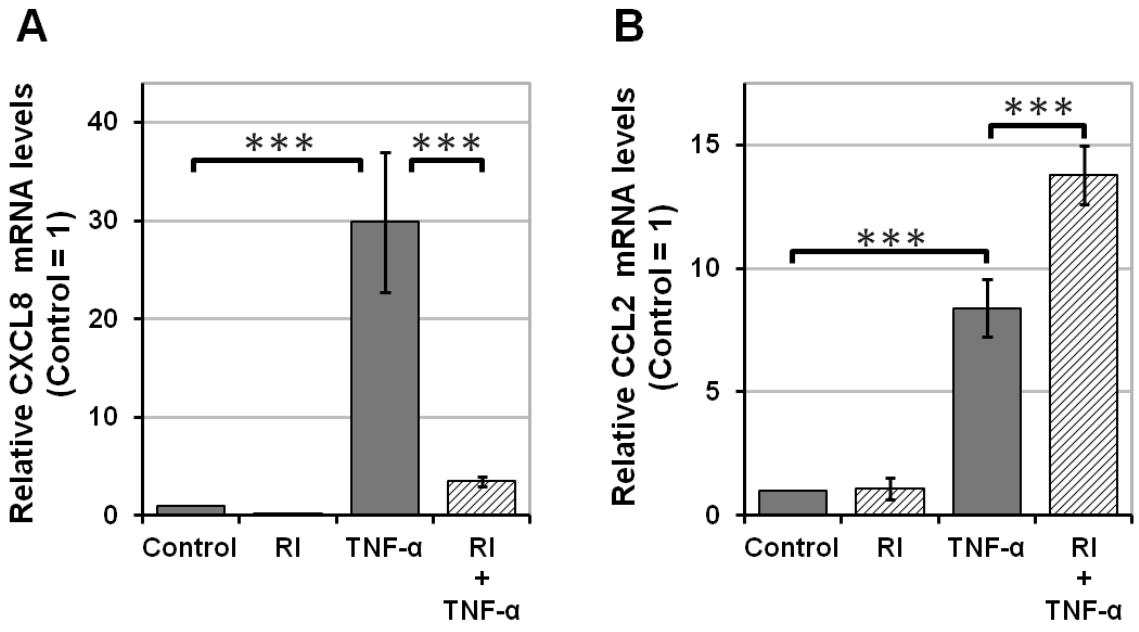

Figure 3.3: Inhibition of ROCK in TNF-α-stimulated Caco-2 cells results in a significant reduction in CXCL8 mRNA expression and a significant increase in CCL2 mRNA expression. Caco-2 cells were plated and pre-treated with 40 µM ROCK inhibitor as described in the Materials and Methods. The cells were then treated with 10 ng/mL TNF-α, incubated for 6 h, and lysed. Total RNA was extracted from each sample and analyzed by real-time RT-PCR for CXCL8 (**A**) and CCL2 (**B**) transcript levels using GAPDH as the reference gene. The graphs display the means (± 1 SEM) of relative mRNA levels normalized to unstimulated controls. (n = 3; *** $p < 0.002$).

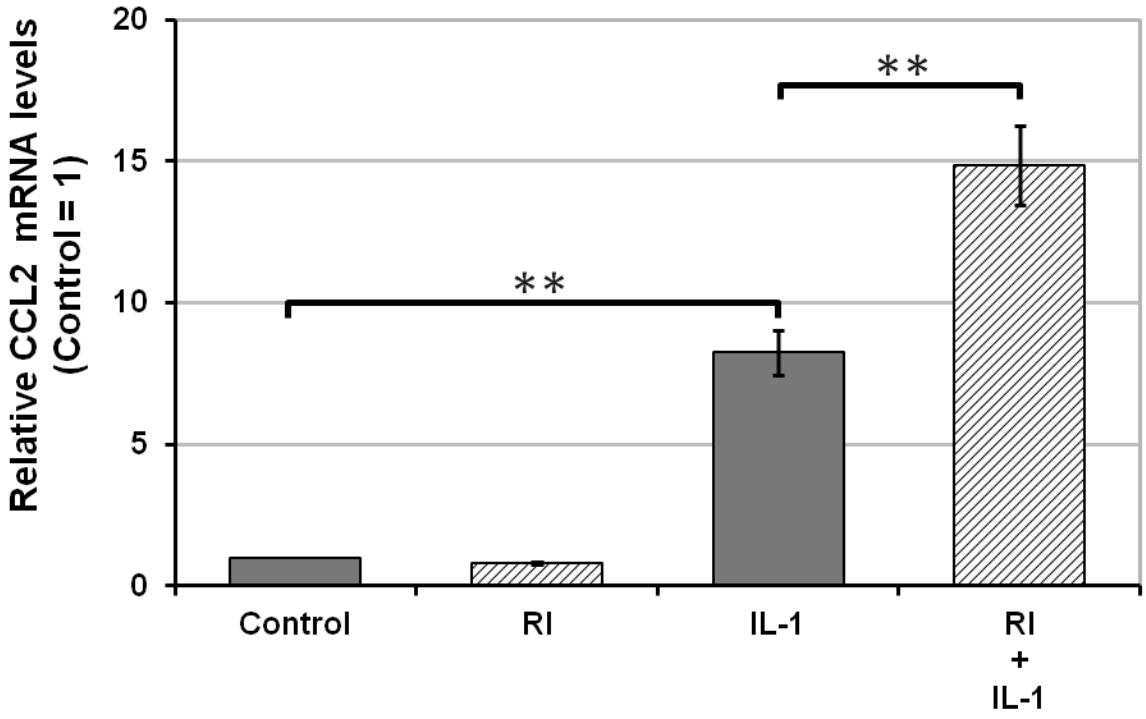

Figure 3.4: Inhibition of ROCK in IL-1β-stimulated Caco-2 cells results in a significant increase in CCL2 mRNA expression. Caco-2 cells were plated and incubated in the presence of the ROCK inhibitor as previously described. IL-1β (1 ng/mL) or plain medium was added to the appropriate wells, and the cells were incubated for another 6 h. Total RNA extracts were then prepared and analyzed via real-time RT-PCR to quantify the relative expression of CCL2 mRNA. The resulting means ± 1 SEM of three independent experiments are presented here. (** $p < 0.0005$).

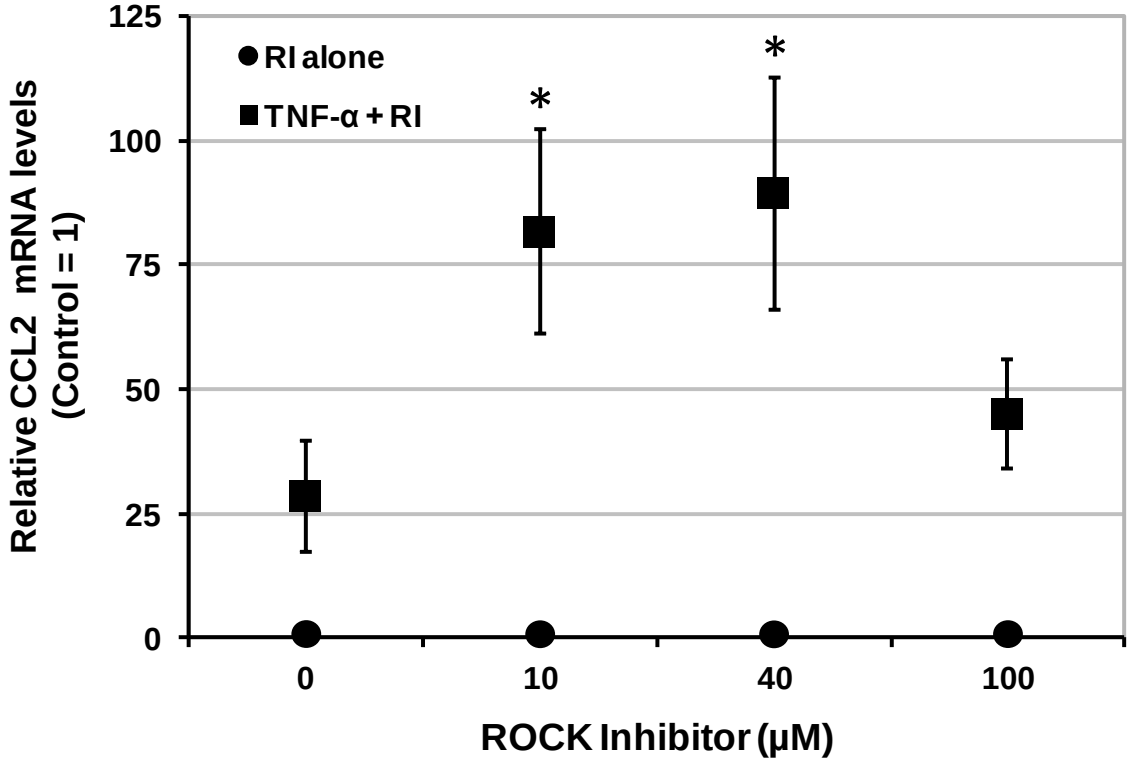

Figure 3.5: The effect of varying the dose of ROCK inhibitor on TNF-α-induced CCL2 mRNA expression in Caco-2 cells. Caco-2 cells suspended in FBS-DMEM were added to the wells of FN-coated culture plates and incubated for 1 hr, allowing the cells to attach. The medium was then removed and replaced with ITS-DMEM containing ROCK inhibitor at the indicated concentrations. After 2 h in the presence of the ROCK inhibitor, the cells were treated with TNF-α (10 ng/mL) and incubated for an additional 6 h. The cells were then lysed, and relative CCL2 mRNA levels were subsequently determined by real-time RT-PCR as described in the Materials and Methods. The means ± 1 SEM from 3 separate experiments are shown here. (* $p < 0.01$ versus cells treated with TNF-α alone).

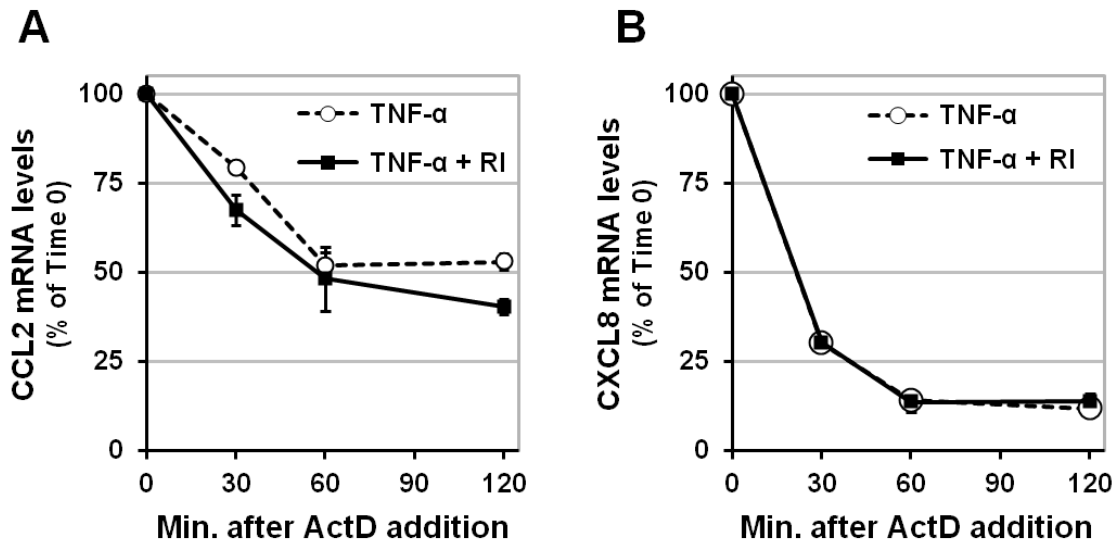

Figure 3.6: The differential regulation of CCL2 and CXCL8 is not achieved through changes in mRNA stability. To examine mRNA stability over time, Caco-2 cells were first subjected to pre-treatment with 40 μM ROCK inhibitor and then treated with TNF-α (10 ng/mL) for 6 h as described in Figure 3.3. At the end of the 6 h, Actinomycin D (ActD) was added to the culture medium at a concentration of 10 μg/mL to halt transcription. The cells were returned to the incubator and lysed after 0, 30, 60 or 120 min of Actinomycin D treatment. RNA was purified from the resulting lysates, and the relative expression of CCL2 (**A**) and CXCL8 (**B**) mRNA normalized to GAPDH mRNA was assessed with real-time RT-PCR. Average mRNA levels expressed as a percents of the zero-time control for each treatment condition are shown in the graphs (n = 2).

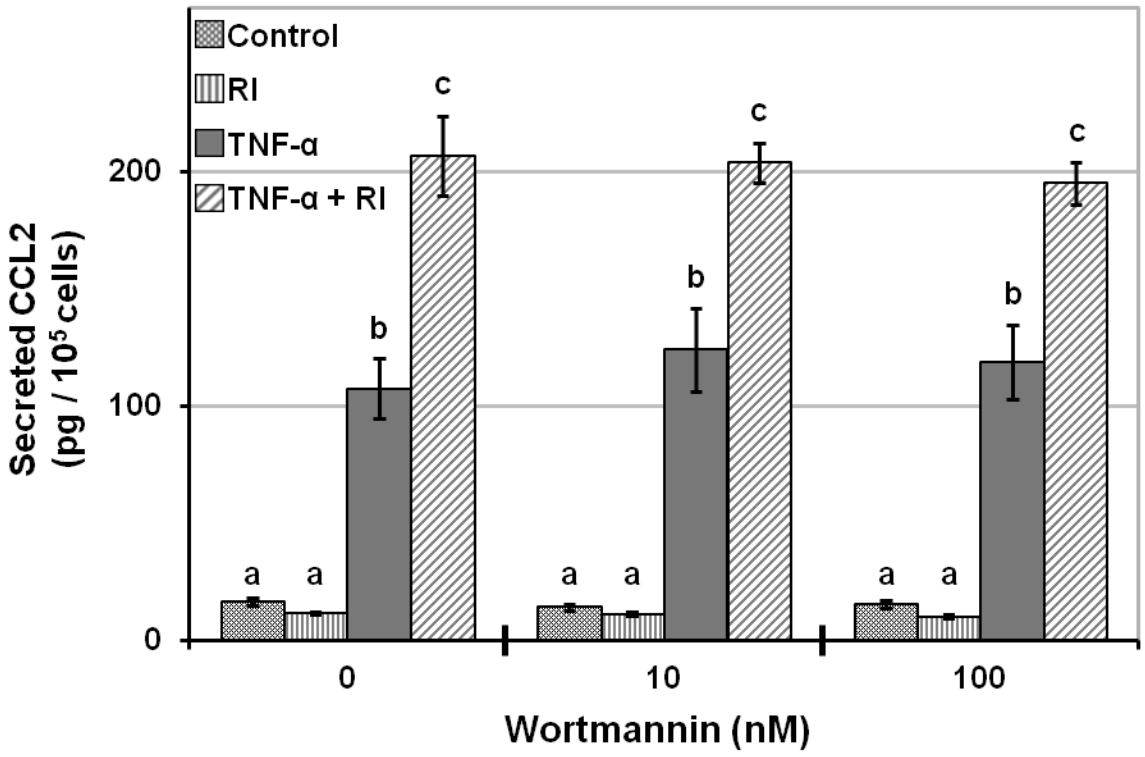

Figure 3.7: The PI3K inhibitor, Wortmannin, has no effect on the enhancement of TNF-α-induced CCL2 secretion that results from inhibiting ROCK. Caco-2 cells seeded in 24-well FN-coated plates were treated with the ROCK inhibitor and 10 ng/mL TNF-α as described in Figure 3.1. However, in these trials, Wortmannin at 10 or 100 nM (or vehicle) was added to the culture medium 1 h prior to the addition of TNF-α. The concentration of secreted CCL2 in culture supernatants collected 24 h after the addition of TNF-α was measured using ELISA and normalized to cells/well. The averages from three separate experiments are shown (± 1 SEM). Letters demarcate groups of statistically similar treatment conditions with each group being significantly different from the others ($p < 0.0001$).

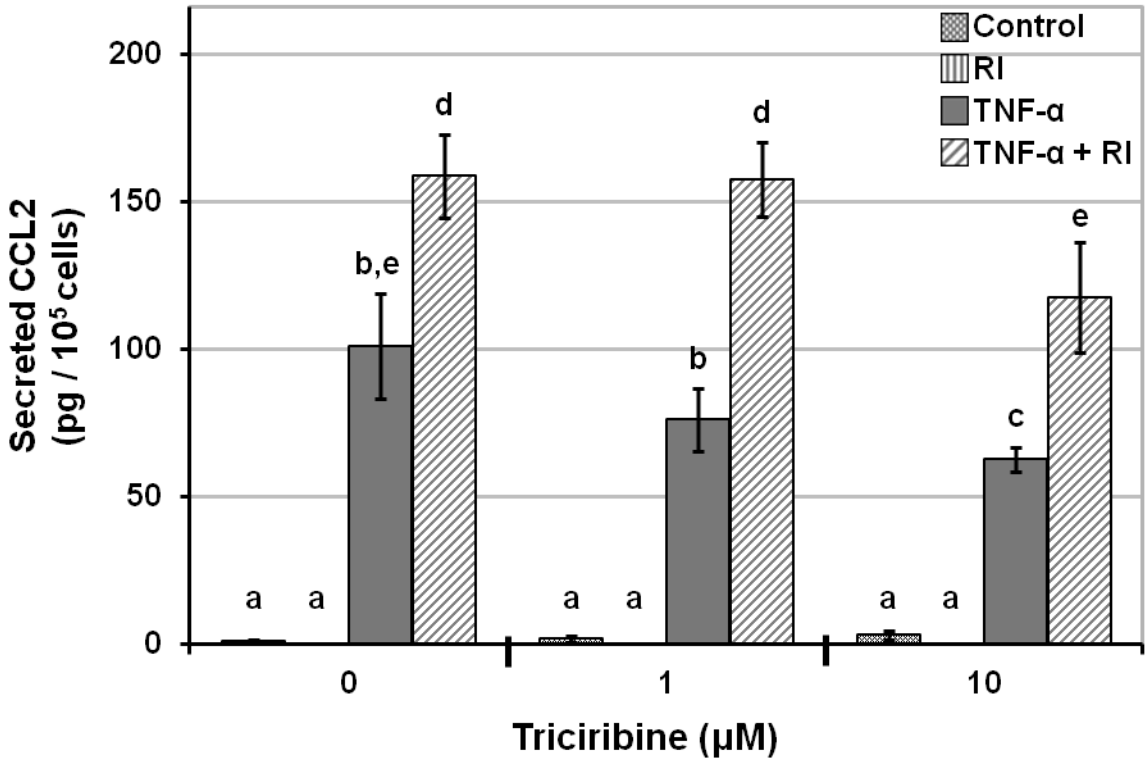

Figure 3.8: The effect of the Akt inhibitor, triciribine, on the enhancement of CCL2 secretion by ROCK inhibition in TNF-α-stimulated Caco-2 cells. Caco-2 cells pre-treated with 40 μM ROCK inhibitor were stimulated with TNF-α for 24 h using the method detailed in Figure 3.1 with the exception that triciribine was added at the indicated concentrations 30 min before TNF-α addition. At the end of the TNF-α treatment, culture supernatants were collected, and the cells in each well were counted. ELISA was used to quantify secreted CCL2, which was then normalized to cells/well. Shown here are the means ± 1 SEM from three independent trials. Statistically significant differences exist between treatment conditions not sharing the same letter label ($p < 0.05$).

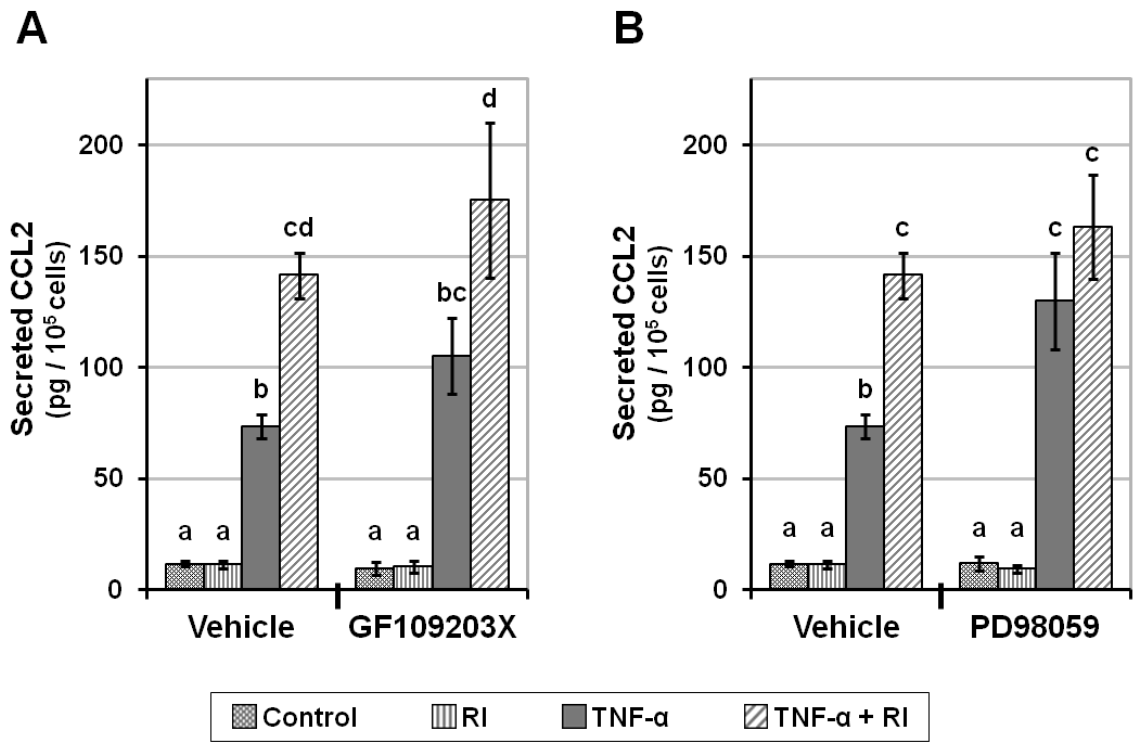

Figure 3.9: The effects of inhibiting PKC or ERK signaling on ROCK-mediated suppression of TNF-α-induced CCL2 secretion. Caco-2 cells were pre-treated with 40 µM ROCK inhibitor and stimulated with 10 ng/mL TNF-α, according to the procedure specified in Figure 3.1, except that 30 min prior to TNF-α addition, the cells were treated with either (**A**) the PKC inhibitor, GF109203X, at 2.5 µM, (**B**) the MEK inhibitor, PD98059, at 25 µM (or vehicle). After 24 h of TNF-α stimulation, CCL2 secretion was evaluated by ELISA. Means ± 1 SEM of three experimental replicates are displayed above. In each replicate, both GF109203X and PD98059 treatments were performed alongside a single set of vehicle-only controls. The vehicle-only data are shown twice for ease of comparison. In each graph, treatment conditions not sharing a common letter label are significantly different ($p < 0.01$).

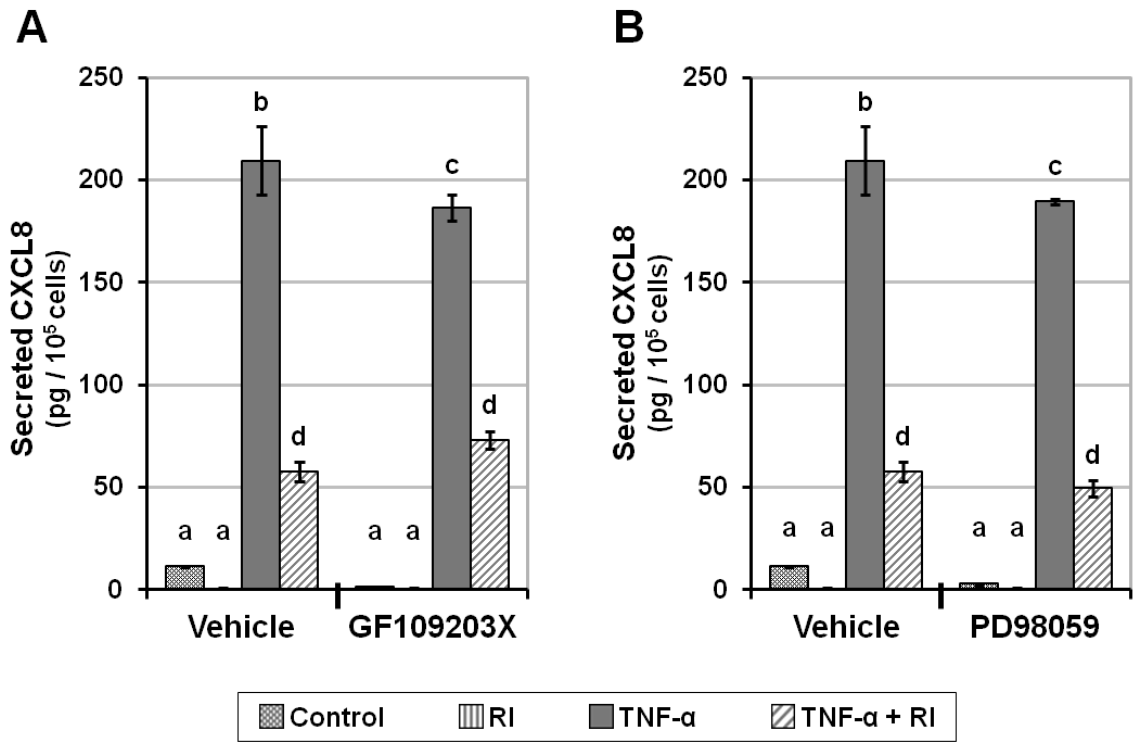

Figure 3.10: The effect of inhibiting PKC or ERK signaling on the suppression on CXCL8 secretion that results from inhibiting ROCK in TNF-α-stimulated Caco-2 cells. Caco-2 cells were pre-treated with 40 µM ROCK inhibitor, treated with either (**A**) the PKC inhibitor, GF109203X, at 2.5 µM or (**B**) the MEK inhibitor, PD98059, at 25 µM (or vehicle), and then stimulated with TNF-α for 24 h as described in Figure 3.9. Culture supernatants from the three independent experiments were analyzed for CXCL8 content using ELISA, and the resulting concentrations were normalized to cells counts. As in Figure 3.9, data for the vehicle-only controls are shown twice for ease of comparison. In each graph, letters indicate groups of statistically similar treatment conditions. Differences between groups are statistically significant ($p < 0.05$ for group b vs. c, $p < 0.001$ for all other comparisons).

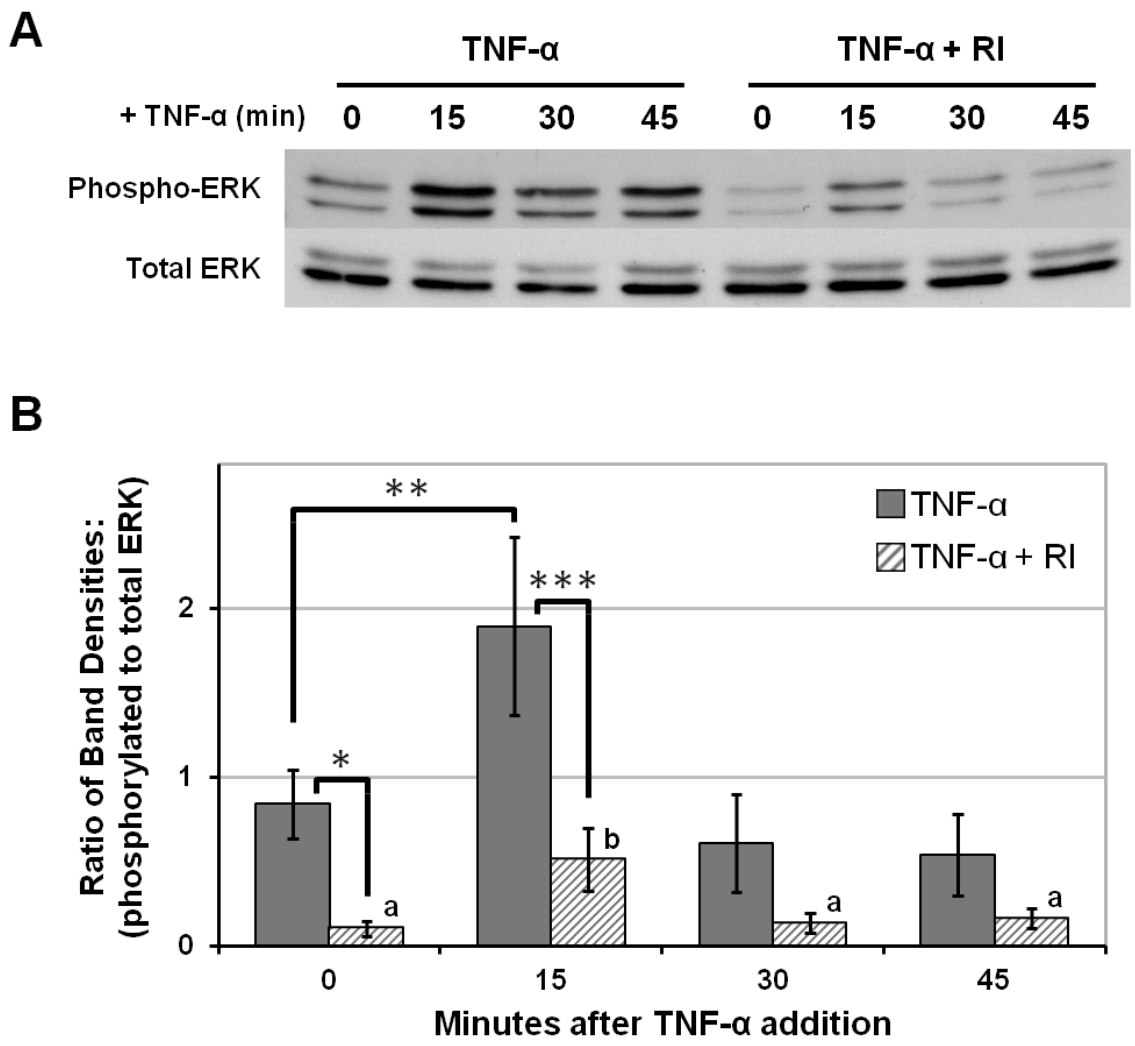

Figure 3.11: ROCK inhibition leads to the suppression of ERK phosphorylation. FN-coated 12-well plates were seeded with Caco-2 cells and incubated for 1 h to allow the cells to attach. The cells were then pre-treated with 40 µM ROCK inhibitor for 2 hr as described in the Materials and Methods, followed by TNF-α (10 ng/mL) treatment for the indicated times. Cytoplasmic proteins were separated via SDS-PAGE and analyzed by Western blot. Blots were probed for phosphorylated ERK1/2 and total ERK1/2. (**A**) A representative blot. (**B**) Graph displaying the means (± 1 SEM) of the ratios of phosphorylated ERK band densities to total ERK band densities (n = 5; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Letter labels denote groups within the TNF-α + RI data subset determined to be significantly different by ANOVA with Fisher's PLSD (group a vs. group b: $p < 0.05$).

4. Discussion

Several chemokines are found at abnormally high levels in IBD and thus likely play important, and perhaps critical, roles in the pathophysiology of IBD. IEC are important components of mucosal immunity and are known to produce chemokines in response to pro-inflammatory signals. Understanding how chemokines are regulated at the mucosal surface may provide opportunities to control the recruitment of leukocytes into affected tissues and thus to control the progression or the maintenance of the chronic inflammation that characterizes IBD.

Previous results in our laboratory have indicated that ROCK is required for optimal production of the neutrophil chemoattractant, CXCL8, in cytokine-stimulated IEC [176; Chapter 2]. Results in this study show that ROCK plays yet another role in regulating chemokines in IEC. Unlike the suppressive effect of ROCK inhibition on CXCL8, inhibiting ROCK in Caco-2 cells treated with TNF-α or IL-1β resulted in an enhancement of secretion and mRNA levels of CCL2 and CCL20, two C-C chemokines that are also implicated in the pathophysiology of IBD. Furthermore, the relative enhancement of secretion seen with ROCK inhibition closely mirrored the enhancement of mRNA levels. Our results also showed that this enhancement of mRNA did not occur through a reduction in the mRNA decay rate. Therefore, it appears that ROCK activation may suppress CCL2 and CCL20 responses in these cells by reducing mRNA levels through the downregulation of transcription. Subsequently, our studies suggest that the effect of ROCK signaling that controls the production of CCL2 in TNF-α-treated Caco-2 cells may be transduced through the ERK pathway, as inhibiting MEK had the same enhancement effect on TNF-α-induced CCL2 secretion as did inhibiting ROCK, while

inhibiting ROCK resulted in a significant decrease in TNF-α-induced ERK phosphorylation. Therefore, a working hypothesis would be that TNF-α activates ERK through the action of ROCK and then ERK subsequently suppresses the TNF-α-induced mRNA expression and secretion of CCL2. However, the mechanism by which ERK affects CCL2 production is still unclear.

To our knowledge, this is the first report of a suppressive regulatory role for ROCK in the production of pro-inflammatory cytokines. As recent research has begun to focus more attention on ROCK and, in particular, its role in chemokine production, there have been reports of ROCK-dependent CCL2 regulation in LPA-treated HUVECs [209], angiotensin-II-treated human hepatic stellate cells [259], TNF-α-treated mouse mesangial cells [260], and IL-1β-treated rat IEC-6 cells [176]. Less is known about the role of ROCK in CCL20 production, although one study found that CCL20 mRNA expression was dependent on ROCK activity in human articular chondrocytes exposed to repetitive compression [261]. In all these cases, ROCK was shown to be a required, positive regulator of CCL2 or CCL20 production. The suppression of CCL2 and CCL20 by ROCK in IEC is a novel finding and may be exclusive to IEC, or perhaps to all barrier cell types, possibly providing a mechanism for finer control of immunological responses in areas with a high degree of contact with a multitude of foreign antigens and organisms.

That inhibiting ROCK resulted in an enhancement of CCL2 and CCL20 responses was a surprising result, which ran contrary to our initial hypothesis. After this effect was confirmed at the mRNA level, strong evidence against the possibility that the enhancement was due to an off-target effect of the Y-27632 inhibitor was obtained from dose-response experiments. These results, taken together, indicate that it is likely that

ROCK is an essential component of a negative regulatory mechanism that suppresses CCL2 and CCL20 production. Furthermore, the fact that ROCK inhibition enhanced CCL2 and CCL20 responses while simultaneously suppressing the CXCL8 response suggests that ROCK mediates at least two independent downstream regulatory pathways governing chemokine responses in IEC. However, the Y-27632 ROCK inhibitor can affect other kinases, and to show absolutely that the effect on CCL2 and CCL20 secretion was due to ROCK, RNA interference using small interfering RNAs (siRNAs) to knockdown ROCK levels must be done.

To characterize the role of ROCK in IEC chemokine responses, we next focused our investigations on the mechanisms underlying the ROCK-mediated regulation of CXCL8 and CCL2. Data from this investigation suggest that the mechanism by which ROCK moderates the secretion of CCL2 in IEC is through the down-regulation of signal transduction and transcription. When ROCK was inhibited in Caco-2 cells, CCL2 mRNA levels increased 1.7-fold in TNF-α-treated cells (Figure 3.3B) and 1.8-fold in IL-1β-treated cells (Figure 3.4). These increases corresponded to respective 2-fold and 1.5-fold increases in CCL2 secretion. While the comparison of the CCL2 transcript levels at 6 hours to the levels of secreted CCL2 protein accumulated over 24 hours does not definitively rule out translational regulation, the fact that ROCK inhibition resulted in equivalent increases in both mRNA levels and secretion levels makes it unlikely that the ROCK-dependent attenuation of CCL2 was occurring through the translational mode of control. Any suppression of CCL2 translation by ROCK would have amplified the suppression of mRNA expression resulting in levels of CCL2 secretion significantly lower than those observed.

The contention that ROCK-mediated suppression of CCL2 in IEC most likely occurs only through transcriptional regulation, but not post-transcriptional regulation, is further supported by results from our experiments examining mRNA stability. Despite the fact that the modulation of mRNA stability is one of the major ways that chemokines are regulated [242], in our experiments, the inhibition of ROCK in TNF-α-treated Caco-2 cells did not lead to increased stability of CCL2 mRNA nor to decreased stability of CXCL8, as would be expected if ROCK were exerting an effect in this way. It should be noted that blocking ROCK did produce a decrease in the stability of CCL2 mRNA at the 2-hour time point. However, this runs counter to the increase in CCL2 transcript levels seen with ROCK inhibition, since decreased stability would result in reduced mRNA levels. Thus, this difference is probably an artifact of the small sample size (n = 2). While at least one study has implicated ROCK in the modulation of mRNA decay [262], our results indicate that this mode of regulation is not utilized in the ROCK-dependent mechanisms regulating CXCL8 and CCL2 production in TNF-α-stimulated IEC. Furthermore, in previous experiments, the inhibition of ROCK in Caco-2 cells was shown to have no effect on TNF-α-induced activation of p38 MAPK (Figure 2-3), a pathway known to play a major role in regulating both mRNA stability and translational control [205, 206, 263]. Although it has been shown that ROCK has the ability to affect mRNA stability in at least one other system [262], our investigations found no evidence of ROCK-mediated post-transcriptional regulation in IEC, and therefore, our data support a transcription-level-only model of ROCK-mediated regulation of TNF-α-stimulated CXCL8, CCL2, and CCL20 production.

Our previous experiments showed that the TNF-induced activation of the NF-κB and p38 MAPK pathways in Caco-2 cells occurs independently of ROCK under our conditions, while JNK was found to be a downstream effector of ROCK in TNF-α-stimulated cells (Chapter 2). Since JNK is a well-known mediator of CXCL8 transcription [194, 197-200], it seemed unlikely that this kinase would also be involved in the suppression of CCL2, and thus, we continued our search for other TNF-α-activated and ROCK-mediated pathways. While several reports have linked the PI3K/Akt pathway to the regulation of CCL2 [153, 154, 256, 264, 265], in our experiments, neither inhibiting PI3K with Wortmannin nor inhibiting Akt with triciribine had any effect on the ROCK-mediated enhancement of TNF-α-induced CCL2 secretion. This result, however, was not surprising, since CCL2 production is not always regulated via PI3K/Akt [266, 267]. Still, it is possible that Akt may play a minor role in regulating TNF-α-stimulated CCL2 secretion independently of ROCK, as treatment with the highest dose of triciribine (10 μM) did produce a modest, overall decrease in TNF-α-induced CCL2 levels regardless of whether ROCK was inhibited. Similarly, the PKC inhibitor, GF109203X, had little or no effect on the ROCK-dependent CCL2 attenuation in TNF-α-stimulated Caco-2 cells.

The first evidence identifying one of the signaling molecules that partner with ROCK in suppressing CCL2 came from treating TNF-α-treated Caco-2 cells with the PD98059 inhibitor. This inhibitor is highly specific for MEK1/2, the only known activators of ERK1/2 [168]. In our experiments, the blockade of MEK, and hence ERK signaling, resulted in enhanced TNF-α-induced CCL2 secretion. A comparable effect was also demonstrated by Pastore *et al*. [268] who found that TNF-α-induced expression

of CCL2, CCL5, and CXCL10 was likewise enhanced by inhibiting ERK signaling in keratinocytes. In addition, over-expression of ERK in HUVECS has been found to suppress VCAM-1 and ICAM-1 expression induced with either TNF-α or IL-1β [269]. In our experiments, the enhancement of CCL2 seen with the inhibition of MEK was nearly identical to the enhancement seen with the inhibition of ROCK as well as to the enhancement seen with the co-inhibition of ROCK and MEK together. That inhibiting ROCK, inhibiting MEK, or inhibiting both in tandem produced such similar regulatory outcomes suggests that ROCK and ERK may be required factors in the transduction of the signal that directs the attenuation of TNF-α-induced CCL2 secretion. Moreover, inhibiting MEK had little or no effect on TNF-α-stimulated CXCL8 responses, which indicates that the MEK/ERK pathway is part of a CCL2-focused signaling branch that diverges from the pathway regulating CXCL8.

This appears to be a unique finding in epithelial cells as other studies of the regulatory effects of ERK on both CCL2 and CXCL8 expression in different epithelial cell lines and primary cultures have found these chemokines to both be under the control of ERK. For example, ERK signaling has been found to suppress TNF-α-induced CCL2 secretion in keratinocytes, much like we observed in Caco-2 cells, while ERK was also found to simultaneously enhance CXCL8 secretion in those cells [268]. Thus, while the overall pattern of differential CCL2 and CXCL8 regulation is parallel to that in Caco-2 cells, the divergence of regulatory signals in keratinocytes occurs downstream of ERK, since the activation of ERK was required for both effects, a finding that is in contradiction with our observations in Caco-2 cells. More commonly, both CCL2 and CXCL8 have been found to be positively regulated by ERK. Experiments with human

peripheral blood mononuclear cells, corneal fibroblasts, renal proximal tubule cells, retinal pigment epithelial cells, and several other cell types have all shown that the full and concurrent induction of CCL2 and CXCL8 is dependent on ERK activation in a variety of pro-inflammatory conditions [255, 256, 270, 271]. It is unclear why CCL2, but not CXCL8, would be regulated by ERK in our cells when the regulation of both chemokines by ERK appears to be the norm for most other cell types. Perhaps reduced CCL2 and CCL20 levels confer an advantage in maintaining or restoring the intestinal barrier in pro-inflammatory conditions that favor the activation of ERK, or perhaps regulating CXCL8 independently of ERK allows IEC more freedom to modulate the amount of CXCL8 secreted and thus provides a wider array of possible responses to the many different types of antigens and stimuli at the intestinal border.

Having found the link between ROCK and ERK, we next clarified their relationship by examining the effect of inhibiting ROCK on TNF-α-induced ERK phosphorylation. These experiments identified ROCK as the upstream mediator of ERK activation in our model, which is in accord with many other examples of ROCK-dependent ERK signaling that have been observed in a wide range of cell types and settings. For instance, ROCK inhibition has been found to suppress the TNF-α-triggered activation of ERK in human lung microvascular endothelial cells [207]. Similarly, in the Caco-2 BBe sub-line, the impairment of ROCK signaling by chemical inhibition or by siRNA-silencing was shown to prevent the activation of ERK that resulted from repetitive substratum deformation [272]. While considerably less frequent, there are also some reports of alternative relationships between ROCK and ERK. Results from two such reports show that in bovine pulmonary artery smooth muscle cells treated with

serotonin and in rat vascular smooth muscle cells treated with platelet-derived growth factor, ROCK does not take part in the induction of ERK activity but instead plays an essential role in the transmittance of the ERK signal by mediating the translocation of activated ERK to the nucleus [273, 274]. Conversely, data from other studies have suggested that in some cases, ERK may act as the upstream regulator of ROCK [152, 275]. It is not entirely clear why such variation in ROCK-ERK interactions would exist, but it could help explain the diversity in the ways that different cell types can respond to the same stimulus.

Figure 3.12 summarizes a proposed scheme of TNF-α and ROCK signaling in our system. TNF-α induces the ROCK-dependent activation of JNK and the ROCK-independent activation of NF-κB, both of which lead to increased CXCL8 expression. Simultaneously, TNF-α stimulation activates ERK, which requires ROCK for maximal activation and leads to suppressed CCL2 and CCL20 production. Because TNF-α can activate ERK even when ROCK is inhibited, it appears likely that ROCK is potentiating ERK activation and not mediating the TNF-α signal to ERK.

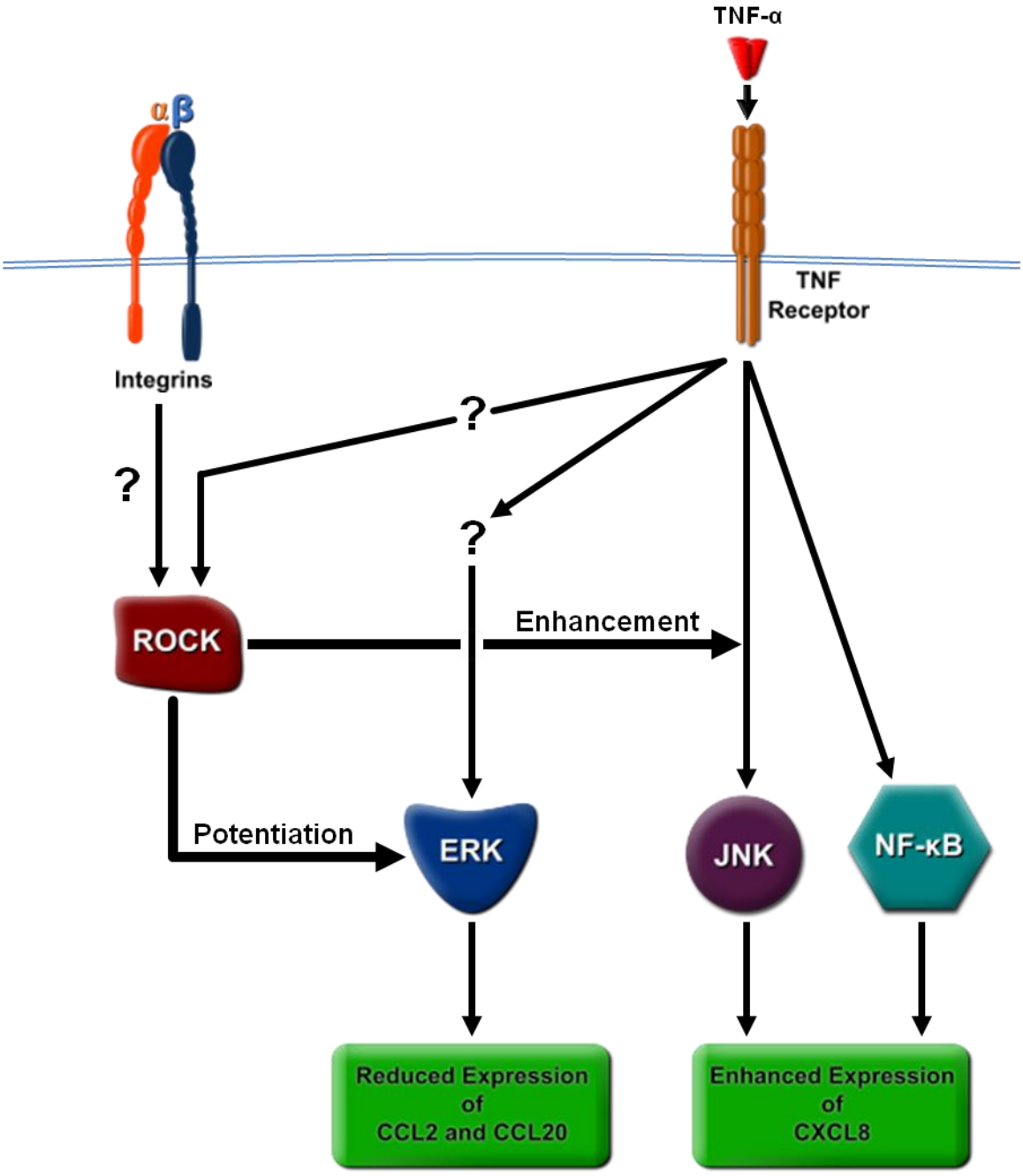

Figure 3.12: Possible modes of TNF-α and ROCK signaling involved in the differential regulation of CXCL8, CCL2, and CCL20 in Caco-2 cells attached to a FN substratum.

What is more striking is that our results indicate that ROCK exerts it effect on ERK phosphorylation independently of TNF-α signaling. The blockade of ROCK suppressed the phosphorylation of ERK regardless of whether or not the cells were treated with TNF-α (See Figure 3.11), and ROCK inhibition had no effect on the ability of TNF-α to elicit ERK phosphorylation, nor did it affect the relative increase in or kinetics of TNF-α-induced ERK phosphorylation. So, while these results suggest that ROCK does not directly transduce the TNF-α-to-ERK signal in IEC, it does appear that ROCK activity potentiates ERK signaling and does so in a manner consistent with crosstalk between two independently activated pathways.

The control of simultaneously induced chemokines via separate regulatory mechanisms has been reported for a variety of chemokines in several different cell types. In HT-29 CL19A human colorectal cancer cells, IL-17 treatment has been found to abolish TNF-α-induced CCL5, CXCL10, and CXCL11 mRNA expression while inducing significant increases in CXCL1, CXCL8, and CCL20 mRNA levels [276]. In another study, the blockade of EGF receptor kinase activity in cells stimulated with TNF-α resulted in the concurrent enhancement of CCL2, CCL5, and CXCL10 responses and suppression of CXCL8 responses [277]. Interestingly, the differential effects of EGF receptor inhibition on CCL2 and CXCL8 observed in keratinocytes parallel the differential effects of ROCK inhibition we found in Caco-2 cells, while in the previously mentioned study, both CCL20 and CXCL8 mRNA levels in TNF-α-stimulated HT-29 CL19A cells were found to be up-regulated together, in contrast to our results.

The number and variety of intracellular signaling mechanisms that have been discovered thus far to be involved in modulating chemokine production is evidence of

complex regulatory systems, most likely specific to cell type, that are capable of making both subtle and extreme changes in the array of chemokines released in response to wide ranges of ever-changing conditions. Such systems may be a way for cells to call in particular sets of leukocytes in order to form specialized immunological networks with distinct functions, such as precise responses to specific types of pathogens, the resolution of inflammation, or the maintenance of tolerance. We have shown here that ROCK can differentially affect the production of CCL2, CCL20, and CXCL8 in IEC treated with TNF-α or IL-1β. CXCL8 is a chemokine that attracts primarily neutrophils [99, 108], while CCL2 attracts mainly macrophage and monocytes [99, 101, 108], and CCL20 attracts dendritic cells and specific subsets of T lymphocytes, including T_H17 cells [100, 101, 113]. Since each of these chemokines attracts a distinct subset of leukocytes, these results suggest that ROCK may be an important component of mechanisms governing the profile of leukocytes mobilized during intestinal inflammation, particularly in conditions that favor the activation of ROCK.

As previously described in Chapter 2, we proposed that the *in vitro* model used in these experiments was applicable to IEC at the edges of intestinal ulcerations that occur in IBD. During our experiments, the Caco-2 cells attached, spread, and began migrating toward one another, remaining subconfluent the entire time. These cellular activities require the formation and turnover of focal adhesions and stress fibers, processes that are known to involve both ROCK and integrins [172, 238, 278]. Integrin signaling has been shown to activate ROCK, to activate ERK, and also to potentiate ERK signaling via ROCK [238, 239, 279, 280]. Therefore, it seems probable that, under the given conditions, the degree of ERK activation stimulated by TNF-α in Caco-2 cells may be a

result of the integration of independent signals from both TNF-α- and integrin-associated pathways. However, our data do not give a clear indication of whether the ROCK-mediated signal and the TNF-α signal work additively to produce the observed activation of ERK or whether the ROCK-mediated signal is amplifying ERK signaling in general. Still, the fact that inhibiting ROCK in untreated controls did not completely abrogate ERK phosphorylation seems to favor the latter explanation.

The idea that our experiments tested cells while they were sub-confluent and moving, much like cells at the edge of wounds, in which IEC are in flux as they attempt to migrate and cover the denuded surface, provides a physiological scenario where ROCK is activated. Coupling chemokine secretion to ROCK activation under these conditions may allow the intestinal epithelium to set up gradients of different chemokines that could affect the spatial distribution of leukocytes in the wound area. For example, our results suggest that ROCK activity is required for optimal CXCL8 secretion in IEC, and therefore, it seems likely that the greatest production of CXCL8 would occur at the wound edge where ROCK activity would be the highest and where neutrophils would be most needed to defend against the influx of microorganisms.

Also, in this model, production for CCL2 and CCL20, which we showed to be suppressed by ROCK activity, would be maximized some distance from the wound edge where IEC would not be actively participating in wound closure, yet would be close enough to the wound edge such that there still would be relatively high concentrations of antigens and pro-inflammatory cytokines. In addition to attracting monocytes and macrophages, CCL2 has been identified as a chemoattractant for B cells, activated T cells, and naïve dendritic cells. A gradient of CCL2 that draws these cells near, but not

into, the wound could be advantageous for many reasons. The environment in the wound would be inhospitable due to the cytotoxic activities of neutrophils. Thus, such a CCL2 gradient would allow B cells, T cells, and dendritic cells to activate the adaptive immune response in relative safety and without hindering the neutrophil response, and macrophages would be near enough to the wound to act as phagocytes while still performing their roles as regulators of inflammation and interacting with lymphocytes as antigen-presenting cells. In addition, CCL20 has also been found to attract T_H17 cells, which are considered to be important players in IBD [60, 61, 64]. A gradient of CCL20 centered away from the wound edge may then be important in positioning T_H17 cells around ulcerations and may play an indirect role in maintaining the chronic inflammation of IBD.

Because ROCK is involved in the differential regulation of these chemokines and thus, would be a necessary component for setting up the differential spatial arrangement of chemokine gradients proposed here, the results of this study open up the possibility of targeting ROCK or its downstream effectors in treatments designed to alleviate intestinal inflammation by exerting precise control over the recruitment of different types of leukocytes. The development of such treatments, however, would first require further characterization of the ROCK-dependent regulatory mechanisms involved and a comprehensive survey of chemokine production in IEC to determine the extent of ROCK involvement.

Chapter 4

Conclusions

The root cause of IBD is still unknown, but it now seems likely that it involves a combination of a number of factors, resulting in a loss of tolerance and an abnormal response to commensal intestinal organisms. Among the potential causes that have been linked to IBD are genetic traits at over 70 loci, environmental factors, and changes in gut microflora. The hallmark of IBD pathophysiology is uncontrolled and prolonged inflammation that leads to tissue damage and other serious consequences. As inflammation progresses, neutrophils infiltrate the crypt regions and surrounding tissues and begin secreting several damaging substances, including oxidative species [281, 282]. This leads to the loss of the intestinal epithelium in inflamed areas and to the degradation of the lamina propria extracellular matrix, creating ulcerations. In CD, if ulcers persist, they can develop into perforations of the intestinal wall and into fistulas. The infiltration of leukocytes and subsequent tissue damage is accompanied by abnormal overproduction of several pro-inflammatory cytokines, for instance IL-1β, TNF-α, IL-6, CXCL8, CCL2, and CCL20, and dysregulation of anti-inflammatory mediators, such as IL-10 and IL-1Ra. Despite being the focus of much research, there is still much that is unknown about this aberrant production of cytokines. Thus, understanding how inflammatory mediators are regulated may lead to better understanding of the etiology of IBD, which, in turn, may lead to better treatments or even a cure.

One pro-inflammatory cytokine that is of particular importance in IBD is TNF-α. Not only is TNF-α found at elevated levels in the serum, stool, and tissues of IBD patients, but it is also a major and central mediator of inflammation in general. One of the main functions of TNF-α is inducing of the expression of several additional downstream cytokines, including the chemokines, CXCL8, CCL2, and CCL20. Due to its central role in inflammation, TNF-α has become the main target of the current biologic therapies. However, despite their advanced nature, these therapies have shortcomings. They are only effective for about 40 - 60% of patients, and there is an increased risk for serious infections. Further research into the role of TNF-α in IBD may allow improvement of these therapies or more likely, may lead to new therapies, which may be more effective and have fewer side effects and risks.

Previous work in our laboratory has shown that ROCK plays a role in chemokine responses in IEC stimulated by IL-1β, another cytokine found at elevated levels in IBD [176]. In those studies, inhibiting ROCK led to significantly reduced levels of IL-1β-induced CXCL8 secretion and mRNA expression in Caco-2 cells. Inhibiting ROCK had the same effect on CCL2 secretion and mRNA expression in IL-1β-treated IEC-6 rat intestinal cells. In addition, ROCK inhibition resulted in significantly reduced IL-1β-induced activation of JNK, while having no effect on IL-1β-stimulated NF-κB activation. These results led us to ask whether ROCK plays a similar role in TNF-α signaling and chemokine responses in IEC. In this study, we have demonstrated that inhibiting ROCK resulted in a significantly decreased CXCL8 secretion and mRNA expression in Caco-2 cells. Further investigation showed that, similar to the previous results with IL-1β, ROCK inhibition suppressed p54 JNK activation. Also similar to the IL-1β results,

TNF-α-stimulated signaling to NF-κB activation was unaffected by ROCK inhibition as shown by unaltered IκBα phosphorylation and degradation. These results indicate that ROCK may be an important mediator of CXCL8 responses in IEC stimulated with TNF-α or IL-1β.

It is notable that ROCK inhibition resulted in an incomplete 46% suppression of TNF-α-induced CXCL8 secretion and a similarly incomplete 82% reduction in mRNA levels. This is most likely linked to the fact that ROCK inhibition had no effect on TNF-α-stimulated signaling to NF-κB activation, while having a suppressive effect on p54 JNK signaling. CXCL8 expression is governed by both the AP-1 and NF-κB transcription factors. Since AP-1 is a major target of JNK, it seems probable that the suppression of JNK resulted in the suppression of CXCL8. However, until confirmed by experimentation, it is unclear which pathways and transcription factors are mediating the ROCK-dependent expression of CXCL8 and which are responsible for the unsuppressed portion of its expression. To determine this, ELISA-based assays targeting AP-1 and NF-κB could be used to examine TNF-α-induced transcription factor activation in the presence or absence of the ROCK inhibitor. Additionally, experiments using inhibitors of JNK and NF-κB in combination with the ROCK inhibitor could be run to see which conditions lead to complete suppression of CXCL8 responses in IEC treated with TNF-α.

The fact that ROCK inhibition resulted in reduced TNF-α-stimulated JNK activation, while leaving TNF-α-induced signaling to NF-κB unaffected, may have further implications and may be an important piece of information for future studies. In addition to initiating the expression of many pro-inflammatory genes, NF-κB activation in the intestinal epithelium has been shown to be necessary for intestinal homeostasis and

for the activation of some adaptive immune responses [1]. Thus, any potential treatment that blocks NF-κB activity in IEC may be ineffective or have severe side effects. However, we have demonstrated here that the ROCK inhibitor blocks only JNK signaling. This points to a mechanism by which the production of pro-inflammatory mediators like CXCL8 could be significantly reduced, while leaving NF-κB signaling intact to maintain homeostasis. This makes ROCK a potentially attractive subject for pharmaceutical research. While the Y-27632 inhibitor is not approved for human use, two ROCK inhibitors, fasudil [283, 284] and ripasudil [285], have been approved in Japan for the treatment of cerebral vasospasm and glaucoma, respectively, and more inhibitors are in clinical trials in the United States for conditions other than IBD. The work of identifying ROCK inhibitors that are safe for human use has already been done. Therefore, further research into ROCK in intestinal contexts and subsequent clinical trials are important if we are to take advantage of this novel therapeutic target.

In this study, we have also shown a role for ROCK in CCL2 and CCL20 production in IEC. In these cases, we found that inhibiting ROCK significantly enhanced CCL2 and CCL20 secretion in TNF-α-treated Caco-2 cells, signifying that the activation of ROCK may suppress CCL2 and CCL20 responses in these cells. This was in direct contradiction to what we found for CXCL8 secretion and to a previous result in our lab that showed that CCL2 production was significantly reduced by ROCK inhibition in IL-1β-treated IEC-6 cells [176]. IEC-6 cells, however, are derived from rat intestine, while Caco-2 cells are human colonic cells, and the rat immune system is sufficiently different from that of humans as to possibly explain the discrepancy. For instance, the rat immune system lacks a CXCL8 homolog [286]. We also showed that TNF-α-dependent

CCL2 mRNA expression could be enhanced with ROCK inhibition and that this enhancement was not due to increased mRNA stability. Inhibiting ROCK led to a similar pattern of enhancement of CCL2 and CCL20 secretion and of CCL2 mRNA expression in IL-1β-treated Caco-2 cells as well. In addition, we found that the ROCK-dependent suppression of TNF-α-induced CCL2 secretion was mediated by ERK. Furthermore, ROCK inhibition suppressed both TNF-α-stimulated ERK phosphorylation and baseline levels of ERK phosphorylation independently of TNF-α. However, even with ROCK inhibition, TNF-α was still able to activate ERK but at considerably lower levels (Figure 3.11).

In order for baseline levels of ERK phosphorylation to be suppressed by ROCK inhibition, the effect of ROCK on ERK signaling must originate, at least partially, from a source other than TNF-α. Since ROCK is involved in stress fiber formation and focal adhesion turnover, both of which also involve integrins, we propose that the baseline portion of ROCK activity that was observed in our cells is possibly due to integrin signaling (Figure 3.12). It is known that integrin signaling can activate ERK [238, 279], and one study found that Rho mediated integrin-dependent ERK signaling in NIH 3T3 cells plated on FN [239], suggesting a possible role for ROCK in this process. To determine whether integrin signaling is involved in ERK signaling in Caco-2 cells and what role integrins may play in TNF-α signaling, antibodies that neutralize specific integrins could be used in experiments examining baseline and TNF-α-induced ERK activation. The α5β1 integrin is a particularly good candidate as it has previously been shown to mediate FN-induced activation of ERK in Caco-2 [287] and rat IEC cell lines [288].

Another important result from this study was the differential regulation by ROCK of CXCL8 versus CCL2 and CCL20. Inhibiting ROCK decreased the CXCL8 response while enhancing CCL2 and CCL20 responses, indicating that ROCK activity was required for optimal CXCL8 production while suppressing CCL2 and CCL20 production. This increase in CCL2 and CCL20 with ROCK inhibition may seem to suggest that ROCK inhibitors may not be ideal for treatment as the increase in these chemokines may still draw inflammatory cells into intestinal tissues. However, macrophages, the main targets of CCL2, are also known to play an important role in healing processes as well. Macrophages can produce anti-inflammatory cytokines, such as IL-10 and TGF-β, that direct a transition to healing conditions by 1) repressing the production of pro-inflammatory molecules in both an autocrine and paracrine manner and 2) inducing the expression of anti-inflammatory and healing-related substances, including growth factors, angiogenic factors, and protease inhibitors (which prevent further tissue damage) [54]. Furthermore, the drop in CXCL8 seen with ROCK inhibition would reduce inflammatory conditions by reducing the number neutrophils in the affected tissues, and thus could potentially tip the balance toward anti-inflammatory conditions. Still, more must be known to determine whether or not ROCK inhibition may be an effective therapeutic strategy. For instance, the questions of what other chemokines are regulated by ROCK in IEC and whether that regulation differs between CXC and CC chemokines (or some other delineation) still remain. To investigate these questions, one of the commercially available ELISA-based chemokine arrays could be employed in experiments with IEC treated with TNF-α and ROCK inhibitor.

In this work, we have proposed that our experimental system best models IEC at the edge of intestinal ulceration. Not only did the Caco-2 cells attach to the substratum, spread, and begin migrating toward one another during the course of our experiments, but the cells also remained subconfluent the entire time. We propose that these conditions are much like the edge of ulcerations where the IEC are also subconfluent and also spread and migrate in an attempt to cover the denuded lamina propria. Attachment, spreading and migration are all processes that are known to involve integrins and ROCK. Thus, this model, taken together with our results, suggests that IEC may produce different spatial arrangements of chemokine gradients, as ROCK would be considerably more active at the wound edge. Specifically, since we found that ROCK activity was required for optimal CXCL8 secretion under inflammatory conditions (i.e. in the presence of IL-1β and/or TNF-α), peak CXCL8 production would be expected at the edge of an ulceration (Figure 4.1). In contrast, we would expect TNF-α- and IL-1β-induced CCL2 and CCL20 production to be suppressed at the wound edge since our experiments showed that ROCK activity reduced CCL2 and CCL20 secretion. Unchecked, these gradients would draw neutrophils to the center of the wound where they could 1) mount a defense against invading microbes and 2) cause additional and prolonged tissue damage. These gradients would also draw macrophages, T cells, and other leukocytes to an area some distance from periphery of the ulceration where they could direct the progression of the inflammatory response and mount additional defenses. In this model, disrupting these gradients with a ROCK inhibitor would lead to a drop in CXCL8 and thus a drop in neutrophils at the wound center. In this scenario, a ROCK inhibitor would also increase CCL2 production at the wound edge drawing macrophages closer to where healing

processes would be needed. While this is merely hypothetical at this point, our data plus the work of Segain *et al*. [123] suggest that ROCK inhibitors could be important anti-inflammatory agents that could help IBD patients achieve remission.

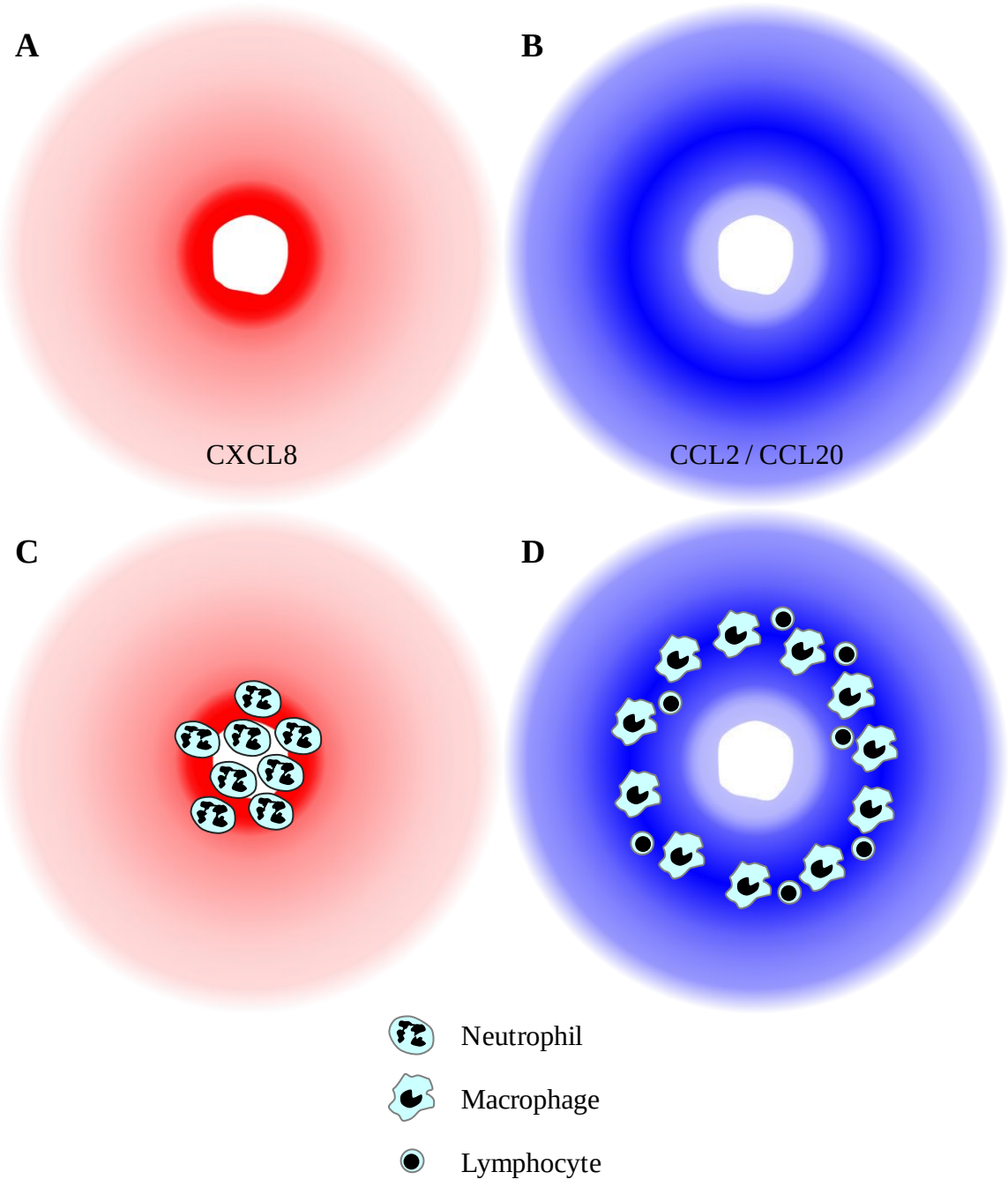

Figure 4.1: Proposed pattern of chemokine gradients produced by IEC at the edge of intestinal ulcerations. **A**) Due to increased ROCK activity in IEC at the wound edge, CXCL8 production (red) would be highest there and decrease with distance from the wound center. **B**) CCL2 and CCL20 production (blue) would be suppressed at the wound edge. Thus, the peak of secretion of both these chemokines would be some distance from the wound edge. A representation is shown here for the expected distribution of **C**) neutrophils and **D**) macrophages and lymphocytes based on the proposed gradients.

While, in this work, we have identified ROCK as an important regulator of chemokine production in IEC and a mediator of TNF-α signaling, many questions still remain about the role of ROCK in IEC inflammatory responses. As previously noted, aside from CXCL8, CCL2, and CCL20, a full inventory of chemokines that are under the control of ROCK has yet to be taken. In addition, much is unknown about the exact position that ROCK occupies in the TNF-α signaling cascade. For instance, the exact Rho protein that activates ROCK has not been identified, nor have any regulators upstream of that Rho, such as guanine nucleotide exchange factors or GTPase-activating proteins. It is also unclear how ROCK fits into JNK signaling. The MAP3K for JNK in TNF-α-stimulated IEC is still unknown, and it is uncertain whether ROCK directly activates a MAP3K or if it regulates JNK signaling by phosphorylating a scaffold protein. Another question is how ROCK potentiates ERK signaling and whether or not integrins are involved. Elucidating these pathways and the full extent of the effect of ROCK in IEC chemokine responses may provide important clues that could either confirm ROCK as a good target for therapeutic intervention or could identify additional therapeutic targets.

In conclusion, we have shown here that ROCK plays a critical role in TNF-α-induced chemokine responses in IEC. Furthermore, we showed that there was a differential effect of ROCK between CXCL8 and both CCL2 and CCL20, although the significance of this is as yet unclear. Still, the ability of ROCK to regulate these pro-inflammatory makes ROCK a good potential candidate for continued pharmaceutical research. In addition, ROCK was also found to regulate JNK, but not NF-κB. This suggests that a ROCK inhibitor drug could reduce IEC inflammatory responses by

suppressing JNK, while allowing NF-κB-dependent IEC homeostasis to go unaffected. Taken together, along with the work of the Segain group [123], our results suggest that ROCK inhibition could be an effective strategy for the induction of remission in IBD.

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